University of Alberta

Development and evaluation of novel liposomal formulations of antisense oligodeoxynucleotides

By

Darrin D. Stuart ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of
the requirements for the degree of Doctor of Philosophy

Department of Pharmacology

Edmonton, Alberta
Fall, 1999

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University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Development and evaluation of novel liposomal formulations of antisense oligodeoxynucleotides by Darrin D. Stuart in partial fulfilment of the requirements of the degree of Doctor of Philosophy.

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ABSTRACT

Antisense oligonucleotides (asODN) can modulate gene expression in a sequence specific manner and therefore hold a great deal of promise in treating many diseases. *In vivo*, free asODN are efficiently filtered by the kidneys and rapidly cleared from the bloodstream and therefore may benefit from a well designed liposomal carrier which can increase delivery of asODN to target tissues. We have developed a formulation which we call coated cationic liposomes (CCL) which are small (<200 nm), targetable, stable in plasma, have a high loading efficiency (80-100%), and are able to circulate in the bloodstream. CCL are first made by optimizing the +/- charge interaction between the cationic lipid and the asODN and then neutral coating lipids are added, including polyethylene glycol modified lipid and coupling lipid, and CCL are formed by a reverse evaporation procedure.

We evaluated the pharmacokinetics of an 18-mer phosphorothioate asODN formulated in CCL following injection into the tail vein of female CD1/ICR mice. Results demonstrate that asODN formulated in CCL have long-circulation characteristics with the majority of the dose eliminated with a half-life of 12 hours and 10% of the injected dose remaining in blood 24 hours after injection. Liver uptake was approximately 30% of the injected dose which is a significant improvement over other cationic liposome/asODN formulations in which >80% of the injected dose distributes to liver.

In order to determine if CCL are able to deliver efficiently asODN to target cells and inhibit the expression of a target gene, we formulated an *MDR1* asODN in targeted and non-targeted CCL and tested their ability to down-regulate the expression of P-glycoprotein (P-gp) in a multi-drug resistant human B-lymphoma cell line. Antibody-targeted CCL delivered approximately three-fold more asODN than non-targeted CCL or free asODN. Results from a functional assay for P-gp suggest that asODN delivered using antibody-targeted CCL results in a decrease in P-gp levels; however, this could not be confirmed by other methods.

These results demonstrate that a well designed liposomal carrier for asODN can have favourable pharmacokinetics *in vivo*, while still maintaining the ability to deliver asODN to target cells *in vitro*.

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LIST OF ABBREVIATIONS

aFGF	acidic fibroblast growth factor
asODN	antisense oligodeoxynucleotides
AUC	area under the time-concentration curve
bFGF	basic fibroblast growth factor
Chol	cholesterol
CCL	coated cationic liposomes
CD19	epitope expressed at the surface of cells of B-cells lineage
cDNA	copy DNA
CPM	counts per minute
DC-Chol	3β[N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol
DDAB	dimethyldioctadecyl ammonium bromide
ddH_20	distilled deionized water
DSPE	distearoylphosphatidylethanolamine
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
DOPE	dioleoylphosphatidylethanolamine
DMRIE	1,2-dimyristoyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide
DOTAP	1,2-dioleoyloxy-3-(trimethylammonio)propane
FBS	fetal bovine serum
Fc	fragment of an antibody molecule that crystallizes
FDA	Food and Drug Administration
GM_1	monosialoganglioside
HBV	hepatitis B virus
HIV	human immunodeficiency virus
HVJ	haemagglutinating virus of Japan
IC_{50}	concentration of drug which inhibits cell growth by 50%

ICAM	intracellular adhesion molecule
i.v.	intravenous
^{125}I-TI	^{125}I-tyraminylinulin
MDR	multidrug-resistance
mRNA	messenger RNA
mAb	monoclonal antibody
MPS	mononuclear phagocytic system
NB	neuroblastoma
PELA	passively entrapped liposomal antisense
PC40	partially hydrogenated egg phosphatidylcholine iodine number 40
pDNA	plasmid DNA
PEG	polyethylene glycol
P-gp	P-glycoprotein
REV	reverse-phase evaporation vesicles
RNA	ribonucleic acid
RSV	Rous sarcoma virus
[S]-asODN	phosphorothioate asODN
TNP	trinitrophenyl
VEGF	vascular endothelial growth factor
V_D	volume of distribution

CHAPTER 1

Introduction and hypothesis

1.1 Introduction

The purpose of the work presented in this thesis was to develop a particulate delivery system for antisense oligodeoxynucleotides (asODN) which would be useful for gene therapy *in vivo*. One of my objectives was to produce a lipid-based formulation which meets the following criteria: be efficient at loading asODN, have a small diameter (<200 nm), be targeted to a specific cell type, be stable and active in biological media (e.g. plasma), and avoid uptake into the liver and spleen upon intravenous (i.v.) administration. The therapeutic use of asODN is complicated by non-sequence specific effects, toxicities, unfavourable pharmacokinetics and poor uptake into target cells. A carrier may be able to overcome some of these problems and increase the therapeutic activity of asODN in a wide range of applications; however, there is currently no carrier for asODN which meets the criteria described above.

The goal of the Human Genome Project is to determine the genetic sequence of the entire human genome. The information generated will be useful in applications ranging from basic cell biology to the diagnosis of disease. One of the most interesting applications of this information is in gene therapy. Gene therapy can be defined as the introduction of exogenous genetic material (DNA or RNA) into cells for the purpose of altering a physiological process in order to improve the medical condition of a patient. Gene therapy could be divided into two basic approaches: 1) the introduction of a gene which is transcribed and translated into a protein which offers some therapeutic benefit to the outcome of the disease; or 2) the introduction of DNA or RNA into cells which alters the expression of disease-causing genes. One of the greatest obstacles to the success of gene therapy is the delivery of genetic material into target cells. The problem of delivery is further complicated by the necessity for the DNA or asODN to reach the proper intracellular compartment in order to function.

Viruses have evolved to package and deliver efficiently DNA or RNA into cells and therefore have demonstrated some potential in gene therapy. Viruses usually display some level of tropism (cell specificity) or can be engineered to target a specific cell type (1-3). They enter cells via receptor-mediated endocytosis and once inside endosomal or lysosomal compartments, fusion proteins in the viral envelope mediate the release of the viral components into the cytoplasm (4). Some viruses express their genes episomally (e.g. adenoviruses) while some viruses integrate their genome into the host genome (e.g. retroviruses). Integration can be advantageous, since it can result in stable, long-term expression. However, integration can be a serious disadvantage since it could result in the activation of an oncogene, or inactivation of a tumour suppressor gene, depending on where integration occurs (reviewed in (4, 5)). Recombinant viruses presently used in gene therapy are unable to replicate *in vivo* and therefore they are not infectious. However, reversion to the wild-type virus, which may be infectious, is a potential problem which must be addressed in preparing viral vectors for clinical use. There are several other disadvantages of viral vectors for gene therapy. It is often difficult to produce enough virus for clinical applications (4). Viral particles can be immunogenic and therefore repeated administration may lead to an immune response. Furthermore, some viral genes are usually present in the vector and may be expressed along with the transgene, resulting in immune responses against transfected cells. There is also a limit to the size of the transgene which can be inserted into a viral vector (all reviewed in (4, 5)). In addition, viral vectors are not useful for delivering synthetic asODN.

Lipid-based carriers, which may be considered artificial viruses, have also been used in gene therapy and offer several advantages over viral vectors. For example, liposomes are non-immunogenic, liposomal vectors may be easier to prepare, and there is no apparent limit to the size of the transgene which can be delivered. Liposomes may also be useful for the delivery of synthetic asODN. The major disadvantage of lipid-based vectors is that they are not as efficient at transfecting cells and long-term expression is not achieved *in vivo* (5).

Antisense inhibition of gene expression could be considered a type of gene therapy. It refers the inhibition of translation of a target messenger RNA (mRNA) by adding a complementary strand of DNA or RNA which hybridizes the mRNA. There are two basic strategies which can be used; cells can be transfected with a plasmid which transcribes a full or partial length antisense RNA, or cells can be treated with short, synthetic oligonucleotides (15-25 bases) (asODN) which are complementary to the target mRNA. In either case, the antisense RNA or asODN hybridizes the target mRNA and causes translation arrest. To date, asODN have attracted more attention and a large number of clinical trials have evaluated their potential for inhibiting the expression of disease-causing genes. A carrier system for asODN or a plasmid DNA encoding an antisense RNA would be useful, however. However, the focus of this thesis was to develop a liposomal carrier for asODN.

This Introduction will include a review of the development of antisense technology from a tool used in basic science to the current therapeutic applications of asODN. Liposomal carriers have played an important role in the preclinical development of asODN and therefore will be discussed in some detail. The important developments in liposomal drug delivery are also reviewed, since much of this information can be applied to developing more suitable liposomal carriers for asODN.

1.2 Hypotheses, objectives, rationale and significance

One of the hypotheses for this project is that cationic lipids can be used to create a liposomal formulation of asODN which is small (< 200 nm), efficient at loading asODN, stable in plasma, and useful for delivering asODN for gene therapy *in vivo*. In order to be useful *in vivo*, particularly for intravenous application, the formulation should avoid uptake into the liver and spleen (unless these are the target tissues) and circulate in the bloodstream for extended times. To reduce adsorption of serum proteins (opsonins), which may lead to destabilization and uptake into the mononuclear phagocytic system (MPS), the formulation should be charge neutral. We hypothesize that the positive charges from the cationic lipid can be sequestered into the liposome interior along with the asODN, and that neutral lipids, including polyethylene glycol (PEG)-modified lipids, can be used to 'coat' the cationic lipid-asODN particles. If such a formulation could be produced, it may not be as efficient at binding cells or delivering asODN as cationic lipoplexes which are often used *in vitro*. Cationic lipoplexes carry excess positive charge and therefore bind cell membranes quite effectively, due to the presence of negatively charged sialic acid on cell membranes. Therefore, one of my objectives was to create a targeted formulation by coupling a ligand (e.g. monoclonal antibody, peptide, etc.) onto the surface of the carrier which recognizes an internalizing receptor on target cells. Rather than binding cells through non-specific electrostatic interactions, like cationic lipoplexes, a cell-specific ligand will facilitate the intracellular delivery of asODN to the target cell population. Another hypothesis is that such a targeted formulation would be more effective at delivering active asODN than similar non-targeted liposomal asODN, or free asODN.

My objectives were first to develop a liposomal formulation which meets the criteria set out above and to evaluate characteristics such as diameter, loading efficiency, and stability *in vitro*. The next step was to evaluate the pharmacokinetics in mice. I believe that the pharmacokinetics and biodistribution of the formulation will be the most important characteristics in determining its potential usefulness for delivering asODN *in vivo*. A final objective was to test the activity of both targeted and non-targeted formulations *in vitro*, and if a suitable model could be developed, I would test the formulations *in vivo*.

As summarized in the following pages, asODN are unique gene-targeted drugs. In spite of the controversy surrounding their activity and mechanism of action, asODN have found, and will continue to find, applications in medicine. One of the most frequently cited reasons for the lack of widespread success of antisense therapy is that asODN do not reach high enough concentrations in target tissues and cells. Free asODN are rapidly cleared from the bloodstream upon intravenous injection, and even asODN which are modified to be nuclease resistant (e.g. phosphorothioates) are susceptible to degradation. In addition, free asODN do not efficiently cross cell membranes. The rationale for this project is that a liposomal carrier may be able to overcome some of the problems related to the intravenous administration of free asODN, and increase the delivery of asODN to target cells. Liposomal carriers have been evaluated as carriers for asODN; however, for the most part, they have not proved useful *in vivo*. Cationic lipoplexes work very well at delivering asODN *in vitro*; however, they are much less active in the presence of serum, and therefore it should be no surprise that they lack activity *in vivo*. In addition, administration of asODN formulated in cationic lipoplexes results in extensive distribution to the liver, leaving tissues outside of the MPS largely inaccessible. In order to evaluate properly the usefulness of a liposomal carrier for asODN, a formulation which has been designed for *in vivo* applications must be used.

AsODN could be useful in a wide range of diseases, their specificity combined with their relative lack of toxicity could result in a very high therapeutic index. As mentioned above, poor pharmacokinetics and inability to reach significant concentration in target tissues have probably hampered the widespread success of asODN. A carrier system which could increase the concentration of asODN in disease tissues through active or passive targeting could improve their efficacy in a broad range of applications.

1.3 Antisense inhibition of gene expression

Translational arrest by antisense RNA occurs naturally in bacterial systems as a method for the regulation of gene expression (6). However, Paterson *et al.* were the first to demonstrate that translational arrest of a eukaryotic gene, β-globin mRNA, could be induced in a cell-free system by the addition of restriction fragments of the copy DNA (cDNA) for β-globin (7). They found an inverse relationship between the concentration of cDNA restriction fragments and the amount of β-globin protein translated. These findings were confirmed by Hastie *et al.* who examined the kinetics of hybridization, giving further support for the theory that translational arrest occurred as a result of Watson-Crick base pairing between the mRNA and restriction fragments from the cDNA (8). The focus of these early studies in cell-free translation systems was to analyse heterogenous mRNA populations and to identify the genes which encode specific proteins. The use of this antisense mechanism for the treatment of disease was apparently not considered in a list of other possible uses, which included mapping simple genomes, correlating cDNA with the corresponding mRNA and protein, and determining the relative abundance of an mRNA (8).

The therapeutic potential of asODN was first alluded to by Stephenson and Zamecnik in 1978 as a possible treatment for viral infection (9, 10). They used a 13-mer phosphodiester ODN which was complementary to the 5' and 3' end of the Rous sarcoma virus (RSV) genome. RSV-infected chick embryo fibroblast cells incubated in medium containing the asODN showed inhibition of viral replication and cell-transformation. Since the sequence targeted in the RSV genome is responsible for circularization and integration into the host genome, it is unclear whether the inhibition of viral replication was due to translational arrest or inhibition of integration; however, the ODN was shown to inhibit translation in a cell-free system (10).

Other studies in cell-free systems began to shed light on the potential mechanism(s) of translational arrest. Hoeuptle *et al.* suggested that translational arrest induced by ODNs in wheat germ cell-free extract was the result of the inhibition of ribosome migration along the target mRNA (11). However, the authors also noted that translational arrest was only partially obtained in a reticulocyte cell-free system and Minshull and Hunt evaluated the importance of RNase H in these cell-free systems (12). They speculated that the difference in hybrid arrest of translation in wheat germ cell-free systems and reticulocyte cell-free systems was due to a lack of RNase H in the reticulocyte system. When reticulocyte cell-free systems were doped with RNase H, the translational arrest of the target mRNA was consistently good, supporting their hypothesis (12). The mechanism of hybrid arrest in antisense therapeutics is still not absolutely clear. RNase H appears to play a role in most instances; however, its activity is dependent on the type of backbone modification in the asODN (Figure 1.2) (13).

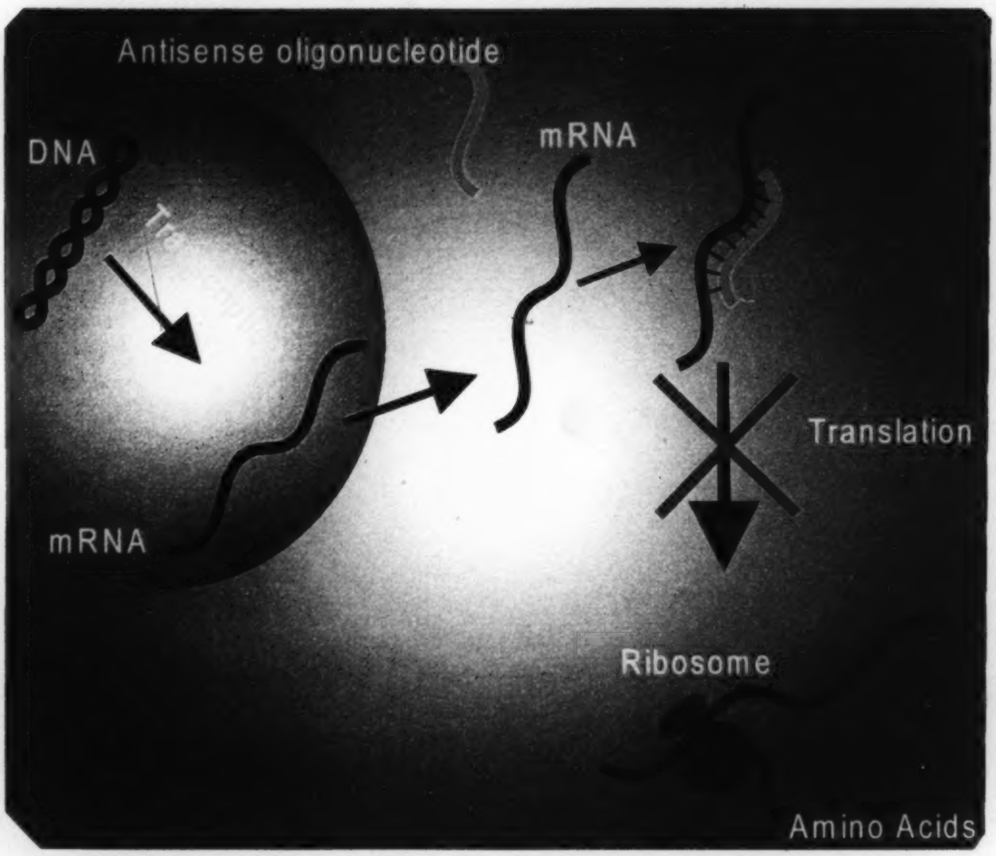
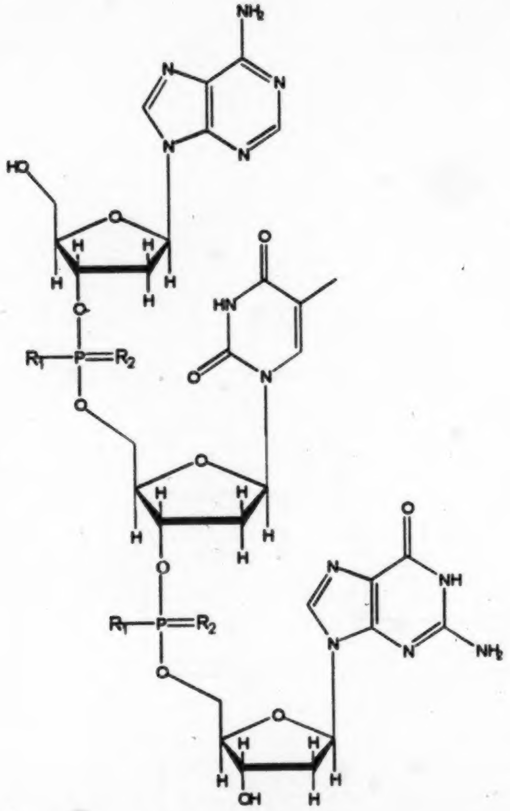

Antisense oligonucleotide
DNA
Tr
mRNA
mRNA
Translation
Ribosome
Amino Acids

Figure 1.1: Diagram illustrating the mechanism of antisense inhibition of gene expression by asODN. Once inside the appropriate intracellular compartment, asODN hybridize the target mRNA through Watson-Crick base pairing. This prevents the translation of the mRNA through inhibition of assembly or migration of the ribosomal machinery, or the activation of RNase H which causes degradation of the mRNA. Ultimately, this will lead to a decrease in cellular levels of the target protein.

Modifications have been made to the phosphodiester backbone of asODN in order to render them resistant to nucleases (13, 14). Typical phosphodiester asODN are susceptible to degradation by nucleases and this significantly decreases their activity. Replacing one, or both of the non-bridging oxygen atoms of the phosphodiester bond with sulphur atoms, results in the formation of phosphorothioate or phosphorodithioate asODN. This modification results in resistance to nuclease digestion but maintains their ability to activate RNase H. Methylphosphonates have also been widely-used. In this case, a non-bridging oxygen is replaced by a methyl group. This also results in nuclease resistance, however, methylphosphonates do not activate RNase H. This is generally considered a negative attribute, since RNase H-mediated translational arrest is highly effective, and results in an actual decrease in the intracellular levels of target mRNA. However, methylphosphonates can be useful tools in cell biology in situations where activation of RNase H is undesirable, such as evaluating pre-mRNA splicing. Conceptually, asODN are a perfect class of drugs for treating disease. The antisense sequence can be tailored specifically to inhibit only the disease-causing gene, leaving normal genes unaffected. This specificity, coupled with the relatively low toxicity of asODN compared to other drugs, should result in a high therapeutic index.

Therapeutic applications of antisense inhibition of gene expression initially centred around antiviral applications and used antisense RNA transcribed from plasmids (15), or synthetic oligodeoxynucleotides (16, 17). However, with the development of synthetic asODN which are modified to be nuclease resistant and can be produced in the large


NH₂
N
N
N
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HO
O
H
H
H
H
O
O
HN
O
N
O
R₁
P
R₂
O
O
H
H
H
H
O
N
NH
N
N
NH₂
R₁
P
R₂
O
O
H
H
H
H
OH

Backbone analogue	R_1	R_2	RNase H activation	Nuclease resistance	Chiral centre
phosphodiester	O^-	O	yes	-	no
phosphorothioate	S^-	O	yes	+	yes
phosphorodithioate	S^-	S	yes	++	no
methylphosphonate	CH_3	O	no	++	yes

Figure 1.2: Properties of frequently used phosphodiester backbone modified asODN. The figure illustrates a trinucleotide with R_1 and R_2 groups replacing the non-bridging oxygen atoms. The table describes the atoms or molecules which are present in R_1 or R_2 for each type of modification. Adapted from (13).

quantities required for clinical applications, the therapeutic potential of antisense ODNs began to be critically examined. Several eukaryote mRNAs (especially oncogenes) have been evaluated as potential antisense targets (reviewed in (18)). There are several other modifications, besides those listed in Table 1.1, which have been made to asODN in order to provide nuclease resistance and/or enhanced uptake into cells (reviewed in (13, 19, 20)). However, the phosphorothioates have found the most wide-spread use in clinical trials.

1.4 Clinical applications of antisense oligodeoxynucleotides

It is difficult to compile a comprehensive list of clinical trials from a literature search; however, *Nature Biotechnology* listed 14 ongoing clinical trials involving asODN in 1997 (21). Very few details are available about these trials, however, it appears that in each case, free asODN was administered either locally or systemically, without using a carrier. In 1998 Isis Pharmaceuticals (Carlsbad, CA) received United States Food and Drug Administration (FDA) approval for Vitravene™, a phosphorothioate asODN ([S]-asODN), for the treatment of cytomegalovirus (CMV) retinitis in AIDS patients (22). This is the first antisense drug to be approved for use in humans and represents the first example of a successful 'gene therapy' product. Isis Pharmaceuticals has also obtained positive results in early phase clinical trials with a [S]- asODN which targets an intracellular adhesion molecule (ICAM-1) (23). ICAM-1 is responsible for leukocyte trafficking and activation and is upregulated in the inflamed mucosa of the bowel in

Crohn's disease. The same ICAM-1 asODN is also in clinical trials for the treatment of rheumatoid arthritis, psoriasis, and renal transplant rejection (21).

The treatment of restenosis following balloon angioplasty has also attracted considerable interest in antisense therapeutics. Genes involved in the proliferation and migration of vascular smooth muscle cells are targeted using asODN (24) and at least one clinical trial has been conducted using this approach (21). There are several oncogenes and other cancer-related genes which have been targeted using asODN (reviewed in (25)) and clinical trials involving asODN to treat Non-Hodgkin's lymphoma, acute myelogenous leukemia, chronic myelogenous leukemia as well as other cancers have been initiated (21).

As mentioned earlier, proof-of-concept of asODN activity was first demonstrated in a model of Rous sarcoma virus infection *in vitro* (9). It may not be a coincidence that the first antisense drug to be approved was an antiviral. AsODNs represent good candidates as antiviral agents due to their intrinsic specificity and also their potential to interfere with different processes in viral infection (integration, replication, translation, etc.). Human immunodeficiency virus (HIV) has been a well-studied target for asODN treatment, and several clinical trials have been initiated to evaluate this strategy (21). As mentioned at the beginning of this section, it is difficult to obtain published information on the progress and findings of many of these clinical trials, and the examples listed above serve to illustrate the wide range of diseases which may ultimately be treatable with asODN.

In spite of the successful therapeutic use of asODN in treating CMV infections, and the positive results obtained in other clinical trials, there are several potential problems with therapeutic use of asODN. The inability of asODN to reach target tissues, target cells, or the appropriate intracellular site of action are major obstacles which must be addressed. In addition, the specific activity of antisense drugs is complicated by significant non-target effects (26).

Non-sequence specific effects are often cited as potential problems with asODN therapy; but very little scientific literature exists on what the causes are. The following examples illustrate a few of the non-sequence effects which have been characterized. [S]-asODN bind to acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) preventing these ligands from binding their true receptors and inducing down-stream effects (27). In addition, [S]-asODN were shown to bind cell surface tyrosine kinase receptors (epidermal growth factor receptor and flk-1) resulting in inhibition of receptor activation (28). A 28-mer phosphorothioate deoxycytidine oligomer was shown to inhibit receptor phosphorylation *in vitro* and suppressed tumour growth in a mouse model of human glioblastoma (28). Rapid infusion of [S]-asODN has been shown cause transient decreases in total white blood cells and neutrophils and cause transient changes in blood pressure (29). These effects are not sequence specific but relate to the type of backbone modification and probably involve interactions with complement proteins (30, 31). Non-sequence-specific effects appear to be extracellular and are concentration dependent. One way potentially to overcome these side-effects may be to use a particulate carrier, such as liposomes, to provide more efficient intracellular delivery, allowing lower doses to be used (26).

Another potential side-effect of asODN (as well as plasmid DNA) in gene therapy is immune stimulation (32, 33). DNA sequences in plasmids, or asODN, which contain unmethylated 'CpG motifs' have been shown activate B cells, monocytes, macrophages, dendritic cells, and natural killer cells (reviewed in (34). The context of the CpG motif in the DNA sequence is important, since some sequences containing CpG motifs are immune stimulating (CpG-S), while other sequences able to neutralize (CpG-N) the stimulatory sequences (34). Receptors for CpG sequences have not been identified, and very little is known about the intracellular signalling pathways which are responsible for these effects (34). Immune stimulation caused by asODN could complicate the interpretation of antisense activity in some therapeutic applications; but CpG-S motifs will be useful adjuvants in DNA vaccines (reviewed in (34)).

As mentioned earlier in this section, the inability to reach therapeutic concentrations of asODN in target tissues has been considered a potential problem (14). Free asODN (administered without a carrier) rapidly distribute out of the blood and into tissues. The major sites of accumulation and metabolism appear to be the liver and kidney with a significant proportion of the dose being eliminated as degraded metabolites in the urine (35, 36). Rifai *et al.* demonstrated that 96% of the injected dose of phosphorothioate ODN was eliminated from blood within the first 30 minutes following injection (37). Most studies indicate that ODNs display a biphasic blood elimination profile, suggesting a two-compartment model, and this is a consistent finding irrespective of the animal model, the ODN, or the method of detection of ODN. The half-life for the distribution phase ($t_{1/2}\alpha$) has been reported to range from less than 1 minute (38, 39), to almost 1 hour (35), and the elimination half-life ($t_{1/2}\beta$) has been reported to range from about 30 minutes (38) to over 35 hours (35). The differences in the absolute values for the α and β half lives may reflect differences in dose, sampling intervals, animal model, method of detection, ODN sequence or the type of modification to the phosphodiester linkage. For example, one study indicated that the clearance rate increased with increasing dose of phosphorothioate ODN and that liver, kidney and spleen uptake decreased at higher doses, suggesting saturation in these organs (37). [S]-ODN are highly protein bound in plasma (98% at 5 minutes) (36) and the apparent volume of distribution has been shown to increase with increasing doses, suggesting that binding to plasma proteins is also saturable (40).

An appropriate carrier for asODN could increase blood levels, prevent asODN degradation, and increase the intracellular delivery of asODN. In addition, tissue-specific targeting of asODN may be possible through the attachment of cell-specific ligands onto the carrier. Cationic polymers such as poly-L-lysine and polyethyleneimine have been evaluated as carriers for asODN (41-43). Both cationic lipoplexes and neutral or anionic liposomes have been more widely used as carriers for asODN than the cationic polymers. The following sections give an overview of the major developments in the field of liposomal drug delivery and are meant to provide some background on liposomes and possibly some insight into how liposomal-asODN carriers may be optimized.

1.5 Discovery and development of liposomes as drug carriers

When phospholipids are mixed in an aqueous medium, they spontaneously assemble into a bilayer structure with the hydrophilic phosphate head groups facing the aqueous medium, and the hydrophobic fatty acid acyl chains forming the interior of the bilayer. Bangham *et al.* made the observation that egg lecithin formed spheres upon hydration in a potassium chloride solution (44) and this appears to be the first published observation of the formation of liposomes. The purpose of the study by Bangham *et al.* was to investigate the permeability of univalent ions through swollen lipid lamellae; however, by the early 1970's 'liposomes' were examined as potential drug carriers (reviewed by Gregoriadis in (45, 46)). Enzymes, polynucleotides, anticancer drugs, steroids, insulin and a variety of other molecules were entrapped within liposomes and studied *in vitro* and *in vivo*.

Early *in vivo* studies evaluating liposomes as drug carriers were encouraging from the point of view that liposomal entrapment of drugs such as penicillin and actinomycin D increased the plasma levels following injection into rats (47). However, it was also found that liposomal drugs rapidly localized to the MPS (47). It was soon recognized that rapid uptake of liposomal drugs into the MPS would make it difficult to target tissues other than the liver and spleen (48, 49); in spite of this, several studies examined the therapeutic potential of liposomal carriers in animal models (50-54). In one of the first published human applications of liposomes, results indicated that ^{131}I-labelled human serum albumin entrapped within liposomes accumulated to a slightly greater extent in tumour tissue compared to normal tissue (55). This observation hinted at the potential for liposomes for delivering chemotherapeutic agents in cancer treatment.

The early studies on the use of liposomes as drug carriers established much of the groundwork and highlighted one of the most important limitations of liposomes as drug carriers; namely that rapid uptake by the MPS would inhibit delivery to other tissues such as solid tumours. Several strategies were found to reduce uptake by the MPS such as the addition of sphingomyelin and/or cholesterol to liposomes (56-59), decreasing the diameter (59, 60) pre-dosing to saturate the MPS (61), or increasing the dose (62). However, a major breakthrough in controlling the MPS uptake of liposomes came in the late 1980's. Allen and Chonn found that the addition of monsialoganglioside (GM_1) to liposomes significantly increased blood levels and decreased MPS uptake (63). Gabizon and Papahadjopoulos confirmed this finding and also noted increased tumour accumulation of GM_1-containing liposomes in implanted tumours in mice (64). These early observations on the Stealth® properties imparted by increasing the surface hydrophilicity of liposomes lead to the development of PEG-modified lipids. Within a very short period, several groups reported the long-circulation characteristics of PEG-containing liposomes (65-69). Presently, PEG is the most widely used stabilizing agent, since GM_1 is more expensive and difficult to purify to pharmaceutical standards (70).

GM_1 and PEG-modified lipids provide steric stabilization to liposomal membranes (see Figure 1.5 for a schematic of sterically stabilized liposome) (71). The hydrophilic moieties which coat the liposomes are thought to inhibit opsonins from interacting directly with the liposome surface. The implications of steric stabilization for liposomes after injection into biological fluids are considerable since adsorption of plasma proteins is thought to play a critical role in the clearance of liposomes from the bloodstream. The link between decreased protein adsorption onto sterically stabilized liposomes and their enhanced circulation time in the bloodstream was demonstrated in a study by Chonn *et al.* (72). They were able to measure plasma proteins bound to liposomes recovered from blood following i.v. injection and their results indicated that GM_1-containing liposomes adsorb less protein and have longer circulation half-lives than formulations without GM_1. Other studies confirm that PEG-coating reduces the adsorption of plasma proteins and results in enhanced circulation times of liposomes in the bloodstream (69).

Reducing the uptake of liposomes by the MPS leads to increased circulation time in blood. The continuous vascular endothelium found in most tissues prevents liposome extravasation, however, increased vascular permeability can occur in tissues under certain circumstances. For example, solid tumours develop their own vasculature in order to grow to a significant size and the developing vasculature has been shown to be leaky (reviewed in (73)). Increased vascular permeability is also a characteristic of tissues which are inflamed, as a result of infection or physical trauma. These two pathological conditions can be exploited by entrapping drugs within long-circulating liposomes which will passively accumulate in these tissues. Liposomal doxorubicin has been shown to be more effective than free doxorubicin in the treatment of solid tumours due to preferential accumulation of liposomal drug in tumour tissue (74). Liposomes are also being evaluated as carriers for antibiotics to treat bacterial infections (75).

In addition to passive targeting, liposomes can also be actively targeted to specific cells by attaching targeting ligands onto the surface. Theoretically, this could result in more efficient liposomal drug delivery to target cells. Furthermore, if the targeting ligand induces internalization of the receptor, this provides an active method of intracellular delivery of the liposomal drug. The following section provides a brief overview of the methods and applications of liposomal targeting.

1.6 Targeted liposomal drug delivery

The idea of attaching ligands onto the surface of liposomes to provide targeting to specific cell types has been considered almost since their inception as drug carriers. Gregoriadis and Neerunjun attached cell-specific IgG molecules to liposomes containing bleomycin and investigated the uptake and subcellular distribution of drug into target cells *in vitro* (49). They found that the cell-specific IgG resulted in a 10-fold increase in the cellular uptake of drug compared to liposomes bearing a non-specific IgG. Others confirmed that antibody-bearing liposomes and their contents could be targeted to specific cell types resulting in increased uptake and activity of liposomal drug *in vitro* (76-80). Studies also evaluated the potential for *in vivo* targeting using immunoliposomes (81); however, the rapid uptake of these conventional formulations by the MPS was considered to be a limiting factor for *in vivo* targeting. This was confirmed by Debs *et al.* when they attempted to target T-cells using conventional immunoliposomes bearing anti-Thy 1.1

monoclonal antibody (mAb) (82). They found that coupling the mAb to the liposomes resulted in a 7-fold increased uptake of liposomes into lymph nodes, probably as a result of binding to resident T-cells; however, there was also an increased uptake into the liver by about 3-fold compared to non-targeted liposomes. They concluded that significant decreases in MPS clearance of immunoliposomes would be required to increase immunoliposomal targeting (82).

The clearance of antibody-targeted liposomes by the MPS could be mediated by Fc receptors present on liver macrophages. Aragnol and Leserman found that dinitrophenyl (DNP)-bearing liposomes were cleared from blood much faster in mice which were immunized against trinitrophenyl (TNP) (83). Fc receptor blockade resulted in increased blood levels and reduced liver uptake of DNP-liposomes in immunized mice. This study indicates that ligand-bearing liposomes can be cleared by the MPS through an immune response against the ligand, and possibly that the enhanced clearance of immunoliposomes observed by Debs *et al.* (82), was mediated by the Fc receptor on MPS cells in the liver.

As described in the last section, steric stabilization of liposomes by GM_1 or PEG-modified lipids results in decreased uptake into MPS tissues. Several coupling strategies have been developed for attaching targeting ligands onto the surface of liposomes, or onto the terminus of PEG-lipids (reviewed in (84)). This has allowed the investigation of *in vivo* targeting using sterically stabilized immunoliposomes (see Figure 1.5 for a schematic of sterically stabilized immunoliposome). Maruyama *et al.* demonstrated that greater tissue targeting is possible when sterically stabilized immunoliposomes are used (85). They found that sterically stabilized (GM_1) immunoliposomes specific for mouse lung pulmonary endothelial cells accumulated to a greater extent in the lungs and had a longer resident half-life in the lungs compared to conventional immunoliposomes. This study also demonstrated that coupling antibodies to sterically stabilized liposomes did not increase the uptake by liver (85).

There have been very few examples which demonstrate a therapeutic benefit of immunoliposomes *in vivo*. However, the studies which have been published use sterically stabilized formulations (86-89). It is difficult to draw any definite conclusions about the feasibility of using targeted liposomes *in vivo*; however, it seems clear is that some method of avoiding MPS uptake is required. Steric stabilization through the use of PEG may be the simplest approach.

1.7 Versatility of liposomes for drug delivery

Liposomes are highly versatile drug carriers for several reasons. Hydrophilic drugs can be encapsulated into the aqueous interior, while hydrophobic drugs can be loaded into the lipid bilayer. Aqueous entrapped drugs can have a great deal of latency, while drugs loaded into the bilayer dissociate quite easily upon dilution in biological fluids. In addition, lipids can be modified to carry a net positive charge on the polar head group, making them useful carriers for DNA or asODN. The following three examples illustrate the versatility of liposomes as drug carriers.

Liposomes which are loaded with a hydrophobic drug are often more like solublizers, than true drug carriers. Good examples are the liposomal formulations of the benzoporphorin photosensitizer drugs (BPD-MA) being developed by QLT PhotoTherapeutics Inc. (Vancouver, B.C.), and currently in phase III clinical trials for macular degeneration. Upon injection, the drug quickly dissociates from the liposomes, probably through transfer to serum lipoproteins. The tissue specificity of drug action is due to the fact that the drug is only active upon irradiation with a laser directed to the blood vessels in the retina.

An example of excellent drug retention by a carrier is when doxorubicin is loaded into the aqueous interior of stable liposome formulations via an ammonium sulfate gradient (90) or pH gradient (91). Very high drug:lipid ratios can be achieved, and using the proper lipid composition, the drug can be effectively retained within liposomes for several days following injection. Experiments have demonstrated that the doxorubicin and the liposomal carrier have identical pharmacokinetics, indicating that drug package is intact, since the pharmacokinetics of the free drug is dramatically different than that of empty liposomes (74). In this case liposomes are acting as true carriers and may enhance the accumulation of drug in sites of disease such as solid tumours (74).

Over the past decade, liposomes have also been seriously evaluated as vectors for the delivery of DNA for gene therapy. Cationic lipids can be synthesized which carry a net positive charge on the polar headgroup (Figure 1.3) (92). Pre-formed cationic liposomes are mixed with DNA and 'lipoplexes' form as a result of the electrostatic interaction between the positively charged lipid and the negative charges on the phosphate groups of plasmid DNA (pDNA). The lipoplexes which are produced often bear no resemblance to liposomes at all and so-called 'spaghetti and meatball' structures form between the DNA and the lipids (93). The lipid protects the DNA from degradation and mediates its intracellular delivery resulting in transgene expression.

These three examples, liposomes as drug solublizers, efficient drug carriers, and DNA vectors, illustrate the versatility of liposomes and highlight their potential uses in medicine. Within each example, there is an extremely wide range of lipids which can be used in order to modulate factors such as the stability, biodistribution, and release kinetics of the liposomal drug. These properties will be considered in the following section which reviews currently used formulations of liposomal asODN.

1.8 Liposomal carriers for asODN

Felgner *et al.* were the first to demonstrate that liposomes composed of synthetic cationic lipids (Figure 1.3) could mediate the delivery of pDNA to cells in culture resulting in measurable transgene expression (92). Later, it was found that cationic liposomes were equally effective at delivering asODN to cells *in vitro*, resulting in dramatic increases in antisense activity (94). Cationic liposomes have become the most widely used lipid-based delivery system for asODN and offer significant advantages over neutral or anionic liposomes. There are several commercial formulations of cationic liposomes available (Gibco-BRL, Boehringer Mannheim, Sigma and others) and all that is required is mixing of the asODN with pre-formed liposomes, followed by a short incubation time at room temperature. Cationic lipid-asODN complexes (lipoplexes) form due to the electrostatic interaction between the positively charged lipid and the negatively charged asODN (Figure 1.4).

Excess positive charge is usually used so that lipoplexes will associate with cell membranes, which carry a net negative charge. Intracellular delivery is thought to occur via endocytosis (reviewed in section 1.9); however, cell-surface receptors recognizing cationic lipids have not been identified. Once inside endosomes or early lysosomes, the DNA must be released into the cytoplasm. Dioleoylphosphatidylethanolamine (DOPE) is often used as a helper lipid in order to facilitate release into the cytoplasm. DOPE has a tendency to form non-bilayer structures, especially at low pH, and this is thought to mediate fusion between the lipoplex and the endosomal membrane, resulting in the movement of DNA or asODN into the cytoplasm (95).

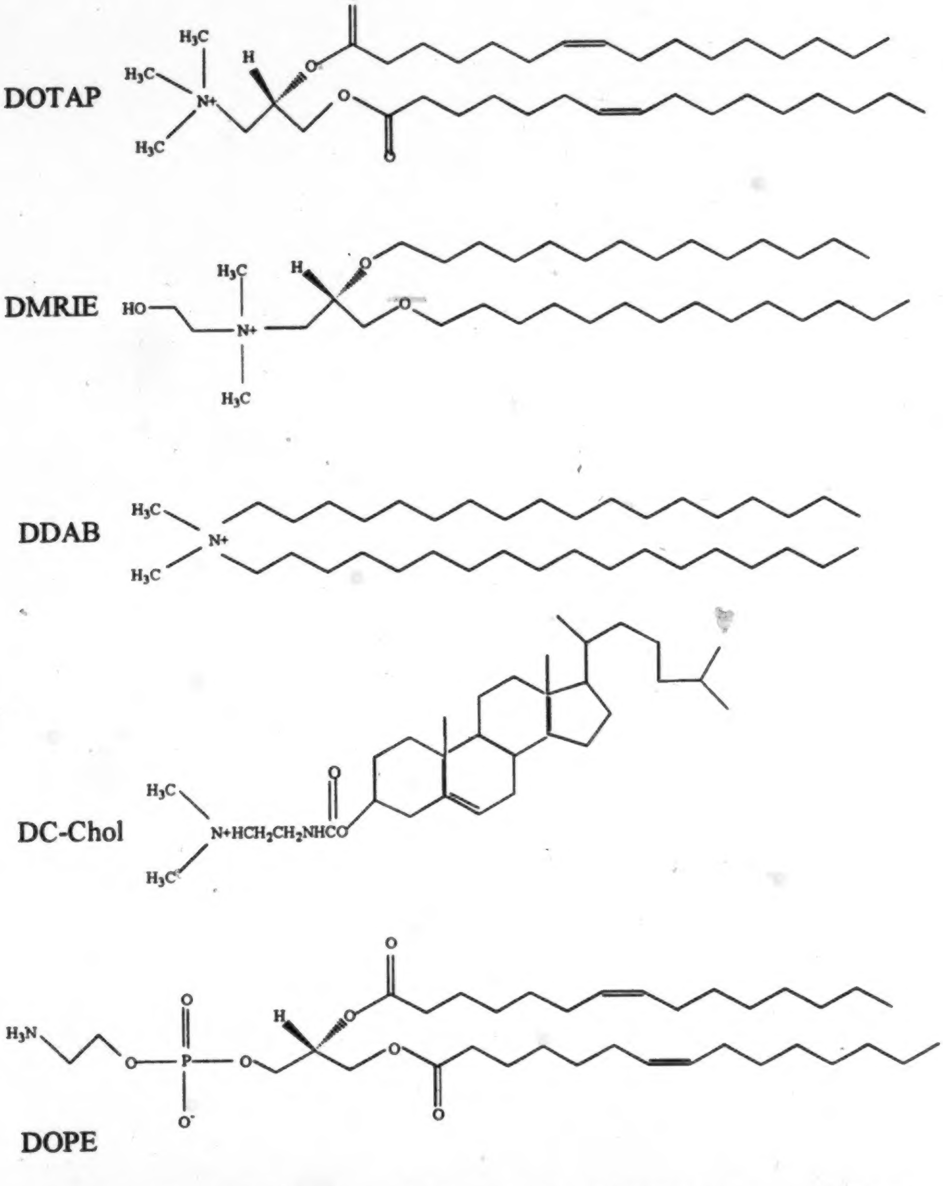
DOTAP
DMRIE
DDAB
DC-Chol
DOPE
H3C
N+
H
O
HO
N+HCH2CH2NHCO
H3N
P
O-

Figure 1.3: Chemical structures of lipids used to produce DNA vectors. DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio)propane; DMRIE, 1,2-dimyristoyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; DDAB, dimethyldioctadecyl ammonium bromide; DC-Chol, 3β[N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol; DOPE, dioleoylphosphatidylethanolamine. Adapted from (96).

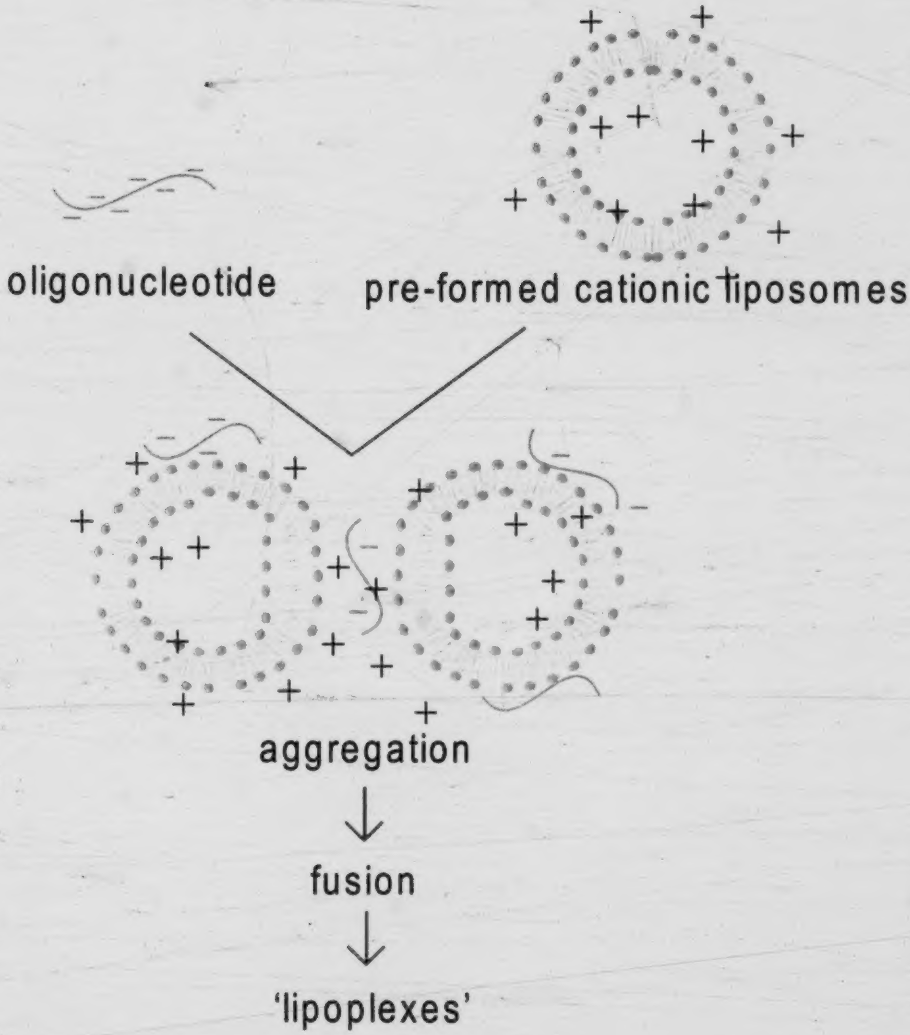
oligonucleotide
pre-formed cationic liposomes
aggregation
fusion
'lipoplexes'

Figure 1.4: Diagram depicting the formation of cationic lipoplexes. Preformed cationic liposomes composed cationic lipid (solid green) and DOPE (open headgroups) are mixed with asODN. Electrostatic interactions lead to aggregation and fusion of liposomes to produce lipoplexes. Adapted from (90).

1.9 Mechanism of intracellular delivery of liposomal asODN

As mentioned in the last section, the most widely used carriers for asODN have been cationic lipoplexes and therefore they will be the focus of this section. The delivery of asODN by cationic lipid carriers can be separated into three basic processes: 1) association with the plasma membrane, 2) cellular uptake, 3) release into the cytoplasm and trafficking to the nucleus.

Cationic lipoplexes carrying asODN or pDNA are thought to interact with cell membranes electrostatically. Cell membranes carry a net negative charge due to the presence of sialic acid residues and lipoplexes should carry an excess positive charge in order to bind to cells (97). This type of electrostatic interaction results in delivery of asODN to a wide range of cells with no apparent selectivity *in vitro*. However, as mentioned earlier cationic lipoplexes are inactive in the presence of serum, and it has been shown that serum alters the zeta potential of lipoplexes from positive to negative, resulting in a decrease in total cell associated asODN (98).

The next step involves the intracellular delivery, which could occur through direct fusion of the lipoplex with the plasma membrane releasing free asODN into the cytoplasm or adsorptive endocytosis of the bound lipoplexes. Endocytosis appears to be the principal method by which lipoplexes enter cells, and evidence for this comes from studies using lipoplexes containing asODN (97), pDNA (99) and empty cationic liposomes (100). Nuclear accumulation of fluorescent asODN (97) and lipid mixing (100) can be significantly inhibited in the presence of endocytosis inhibitors. The mechanism which triggers endocytosis upon lipoplex binding has not been determined.

Once asODN-lipoplexes are internalized into endosomes, the asODN must be released into the cytoplasm or nucleus in order to interact with the target mRNA. As mentioned earlier, DOPE is often used in cationic lipoplexes, and is thought to facilitate the release of DNA from endosomes into the cytoplasm (95). Zelphati and Szoka developed an interesting hypothesis for a mechanism by which asODN may be released from endosomes (101). Their hypothesis is that once the lipoplexes are internalized into endosomes, the cationic lipid mediates a flip-flop of anionic lipids from the cytoplasmic face to the endosomal face of the endosomal membrane. This results in a charge coupling between the cationic lipid and the anionic lipid and induces fusion between the lipoplex and the endosomal membrane, resulting in the release of asODN into the cytoplasm. In order to evaluate this hypothesis in a model system, they added anionic liposomes to cationic lipoplexes in order to mimic what would happen if this flip-flop occurred. They found that anionic liposomes were very effective in releasing asODN from cationic lipoplexes. At a 1:1 charge ratio of anionic lipid to cationic lipid, asODN was readily released from the lipoplexes. This study also provided evidence that fluorescent lipids remain in discrete cytoplasmic structures (endosomes), while fluorescent asODN diffuse through the cytoplasm and accumulate in the nucleus (101). This observation further supports the fusion/release of asODN hypothesis.

Ligands can also be attached to the surface of cationic lipoplexes for the purpose of targeting asODN or DNA to a specific cell type (102, 103). In addition, targeting via an internalizing receptor can provide another mechanism by which liposomal or lipoplex-asODN can enter cells. Once in the endosomal compartment, fusion with the endosomal membrane could occur as described above. However, there is some question as to the ability of a targeting ligand to increase the cell-specific targeting of cationic lipoplexes. The electrostatic interaction between the cationic lipoplex and the cell membrane may override any ligand-specific targeting. In this case, the targeting ligand may be a method to increase the rate of endocytosis of the lipoplex, but probably does not result in specific targeting *per se*.

The final step in the delivery of asODN is trafficking to the nucleus; however, almost nothing is known about how this might occur. It could occur through passive diffusion; but the presence of carrier proteins in the cytoplasm or on the nuclear membrane should also be considered.

This section reviewed the potential mechanisms by which cationic lipoplexes deliver asODN into cells *in vitro*. As mentioned, cationic lipoplexes are relatively inactive in the presence of serum and therefore have normally been used in serum-free medium. Cationic lipoplexes have not demonstrated activity in delivering asODN *in vivo*, partly due to the interference of plasma proteins which may prevent uptake into cells or result in rapid removal by the MPS. Very little is known about the interaction of these carriers with cells *in vivo*, however, the pharmacokinetics have been examined in some detail and are reviewed in the next section.

1.10 Alternative liposomal asODN formulations

The major disadvantage of typical cationic lipoplexes is that they are inactive in the presence of serum, and therefore the activity *in vivo* will be significantly reduced. The potential usefulness of carriers for asODN *in vivo* has recently lead to the development of alternative liposomal formulations of asODN (Section 1.8).

Huang and colleagues have developed methodologies for assembling lipid based carriers for DNA which are referred to as LPDI and LPDII particles (104, 105). Plasmid DNA or asODN are pre-condensed/complexed with poly-L-lysine, followed by the addition of cationic (LPDI) or anionic (LPDII) pH-sensitive liposomes which entrap the poly-L-lysine/DNA complexes. LPDII particles, which probably carry a net negative charge, can be targeted by adding folate-PEG-PE to the composition resulting in increased uptake by cells (over)expressing the folate receptor. Therefore, folate-bearing LPDII particles are taken-up by target cells through a receptor-mediated process, which will be more specific than the electrostatic binding which occurs between cationic lipoplexes and cell membranes. LPDI/LPDII particles have been shown to be active at delivering asODN or pDNA *in vitro*, but there is no evidence for their activity *in vivo*.

Much of the work that has been done optimizing cationic lipoplexes for the delivery of pDNA may also be applied to asODN formulations. For example, replacing DOPE with cholesterol and adding PEG-modified lipid in order to stabilize cationic lipoplexes has been shown to increase the level of transfection following i.v. injection into mice (106, 107). Detergent dialysis methods have also been examined in attempts to produce small, stable lipid-DNA particles which may be more active *in vivo*. The cationic lipid is solubilized in a non-ionic detergent and the DNA interacts with cationic lipid-detergent micelles and following detergent removal, liposomal-DNA particles form. Results from these studies indicate that cationic lipid-DNA particles assembled in this way have some transfection activity in the presence of serum (108, 109). Methods for producing hydrophobic cationic lipid-DNA particles are also being developed which may be useful as intermediates in the formation of lipid-based carriers (110, 111).

Since asODN are hydrophilic, they can also be entrapped within the aqueous interior space of non-cationic liposomes and early studies used asODN passively encapsulated within neutral or anionic liposomes (112-114). This type of formulation offers certain advantages over the cationic lipoplexes described above. Small, stable liposomes can be created which may be quite similar to liposomal formulations of other drugs (e.g. doxorubicin), which have been optimized to circulate for extended times (Figure 1.5, addition of PEG-lipid). As mentioned earlier, cationic lipoplexes bind cells due to the electrostatic interaction, therefore, neutral or anionic liposome formulations will probably associate with cells to a lesser extent. In order to increase cell binding, mAb can be attached to the surface of liposomes, and this will substitute for the electrostatic interaction which occurs with cationic lipoplexes (Figure 1.5).

To increase further the uptake and cytoplasmic delivery of asODN, 'virosomes' can be produced by adding viral proteins to neutral or anionic liposomes. One example which has been published extensively is the HVJ (haemagglutinating virus of Japan) liposome method (24, 115-118). DNA or asODN are passively encapsulated within anionic liposomes which are then mixed at 37°C for one hour with inactivated HVJ. Free HVJ is then separated, leaving fusogenic HVJ-liposomes. The presence of viral proteins in the liposome formulation increases binding to cells, and the presence of the fusogenic protein from the virus increases the cytoplasmic delivery of asODN (Figure 1.5).

HVJ liposomes have only been used for local application *in vivo*, and there is no evidence indicating that they are active following i.v. administration. The presence of viral proteins probably results in an active uptake by the MPS and repeat doses may result in an immune response. One interesting strategy may be to sterically stabilize virosomes by using PEG (Figure 1.5). Viral fusion proteins may be useful for increasing the cytoplasmic delivery of liposome contents and PEG could prevent recognition of the viral proteins by the MPS. Adding a fusion protein to targeted-sterically stabilized liposomes could result in very efficient cytoplasmic delivery if an internalizing receptor is targeted.

The major disadvantage of neutral or anionic formulations is the poor loading efficiency of asODN within small liposomes. This results in low lipid:drug ratios and therefore more lipid is required to deliver a given amount of asODN when compared to a more efficient formulation. AsODN can be passively loaded into liposomes by several different methods including hydrating a dried lipid film in buffer containing asODN, or by a reverse-phase evaporation procedure (REV) (119). Whatever method is used, the loading efficiency will be limited by the aqueous trapped volume and therefore the diameter of the liposomes.

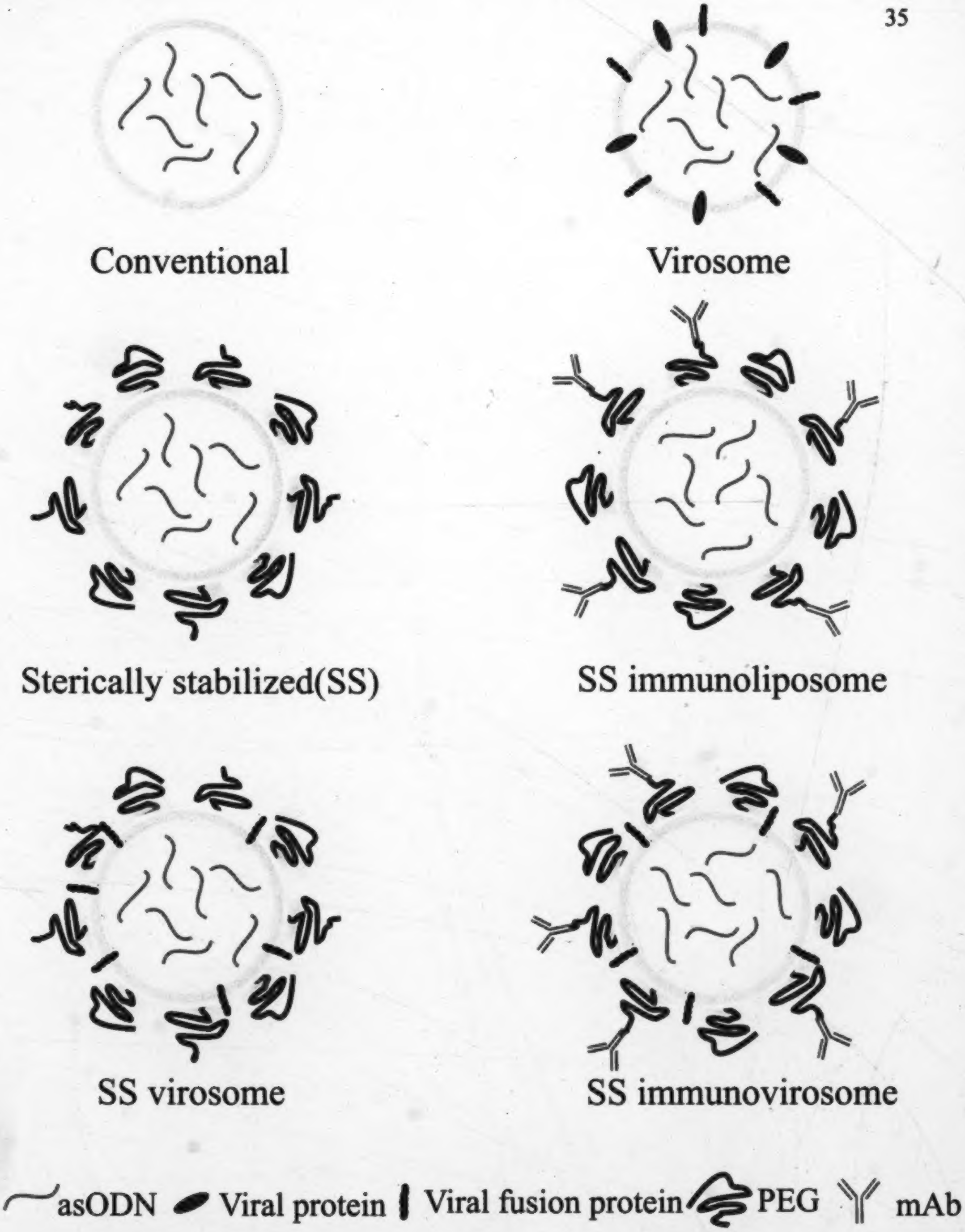
Conventional
Virosome
Sterically stabilized(SS)
SS immunoliposome
SS virosome
SS immunovirosome
asODN
Viral protein
Viral fusion protein
PEG
mAb

Figure 1.5: Passively entrapped liposomal asODN (PELA). The addition of viral proteins will result in virosomes such as the HVJ liposomes; the addition of PEG will result in sterically stabilized liposomes; PEG plus mAb will result in targeted SS immunoliposomes. Virosomes containing viral fusion proteins could also be stabilized by PEG, and targeted using mAb.

1.11 Pharmacokinetics of liposomal antisense oligodeoxynucleotides

Liposomes alter the pharmacokinetics of entrapped drugs, and therefore, could be used to increase the delivery of asODN to certain tissues. Liposomes could protect asODN from degradation, decrease potential toxicities related to free asODN, increase the bioavailability, and provide a mechanism for efficient intracellular delivery. As described in other sections of this chapter, liposomes have the ability to accumulate in diseased tissues such as solid tumours and areas of inflammation. In addition, sterically stabilized liposomes have long residence times in blood offering the potential to deliver asODN to target cells within the vasculature.

Two studies have evaluated the pharmacokinetics of asODN formulated in cationic lipoplexes following i.v. injection into mice. Bennet *et al.* studied the pharmacokinetics of lipoplexes 300 - 500 nm in diameter which were produced by mixing a 20-mer phosphorothioate oligonucleotide with cationic liposomes composed of the cationic lipid DMRIE mixed in a 1:1 molar ratio with DOPE. In a similar study, DMRIE was replaced by DC-Chol and the complexes formed with an 18-mer phosphorothioate oligonucleotide were greater than 1800 nm in diameter. Both studies indicated that the lipoplexes were cleared from blood quite rapidly as a result of distribution to the liver (120) or lung (121). However, accumulation in the lung appeared to be transient since, 20 hours following injection 80% of the initial dose was found in the liver and only 5% remained in lung (121).

Soni *et al.* examined the pharmacokinetics, as well as the therapeutic efficacy, (reviewed in section 1.12), of free and liposomal phosphorothioate asODN in a duck model of hepatitis B virus (HBV) infection (122). The authors formulated a phosphorothioate asODN with cationic liposomes by a dehydration-rehydration method which resulted in liposomes with a final diameter of 200 nm and 50% entrapment of the added asODN. Following injection, the liposomal formulation demonstrated plasma pharmacokinetics which were nearly identical to free asODN over the first hour. Six hours after injection, approximately 10% of the injected dose remained in blood for the liposomal asODN, while 1% remained in blood for the free asODN. Within 1 hour, over 80% of the injected liposomal asODN distributed to the liver, while only 14% of the free asODN was found in the liver at this time. Even though the formulation used in this study is different from cationic lipoplexes, the pharmacokinetics and biodistribution are similar.

The pharmacokinetics and biodistribution as well as the antisense activity of liposomal asODN directed against the *c-raf*-1 oncogene was examined in mice (123). The asODN was formulated into cationic liposomes by hydrating a dried lipid in a solution containing the asODN. The method resulted in high loading efficiency of asODN (90%); however, the liposomal suspension was polydisperse with respect to diameter and included quite large particles (> 2 μm). The pharmacokinetics following injection were similar to the examples described above, with the asODN being rapidly cleared from blood and taken up by the liver, so that approximately 1% of the injected dose remained in plasma 24 hours following injection.

These studies all indicate that asODN formulated into cationic liposomes do not remain in circulation for long periods and are rapidly taken up by the liver. This will undoubtedly hinder the delivery of asODN to other tissues. However, as discussed in previous sections, in order for a liposome composition to avoid immediate removal from circulation by the MPS, it should have a small diameter, be stable in plasma, and have some component of steric stabilization. Based on the observations and lessons learned during 20 years of liposome research, it may have been predicted that lipoplexes with diameters often greater than 1 μm, which carry an excess positive charge, and sometimes contain DOPE, would have very short half-lives in blood. Real progress will be made on developing a good liposomal formulation for asODN when all of these factors are considered and properly addressed.

1.12 *In vivo* applications of liposomal asODN

There are very few examples which demonstrate the effectiveness of a liposomal carrier for asODN *in vivo*, and the poor pharmacokinetics described in the last section may be one of the most important reasons for this lack of success. Only one study has provided evidence to support the use of cationic lipoplexes *in vivo*. Perlaky *et al.* examined the antitumour effect of a [S]-asODN directed against p120 (a human nucleolar antigen) mRNA in human LOX ascites tumour xenografts in nude mice (124). AsODN complexed with cationic liposomes demonstrated significantly greater inhibitory effects on tumour growth than free asODN; however, this was a preliminary report, and follow-up studies have apparently not been published.

The HVJ formulation described in section 1.10 has demonstrated activity for local administration of asODN to tissues such as the myocardium (115), the carotid artery (24), and ligaments of damaged joints (125). However, HVJ liposomes containing asODN or pDNA have not demonstrated activity following systemic administration.

Soni *et al.* used cationic liposomes to deliver an asODN directed against the duck HBV in a duck model of chronic HBV infection (122). As described in the previous section, the liposomes accumulate in the liver following i.v. administration and therefore some level of targeting may have been involved, since hepatocytes were the target cells. There was a great deal of variability in the viral titres of both treated and non-treated animals and although the asODN-treated animals had lower serum viral titres, there is some question as to whether the decreases were a result of a true antisense mechanism.

In the study described above, the authors did not use typical cationic lipoplexes. A lipid film consisting of phosphatidylcholine, DOPE and cationic lipid (stearylamine) was hydrated in a solution of asODN, and the diameter of the resulting liposomes was reduced to 200 nm by a microfluidizer. Using a similar formulation, Gokhale *et al.* administered liposomal *raf-1* asODN i.v. into mice (123). In spite of the rapid clearance from blood and extensive distribution to the liver, there were measurable decreases in Raf-1 protein in tumour xenografts, as well as in normal liver and kidney tissue.

The studies by Soni *et al.* and Gokhale *et al.* represent a change in the way in which cationic liposomes have been used as vectors for asODN. Rather than using pre-formed liposomes composed of cationic lipid and DOPE and making complexes by mixing with asODN, lipids are used which confer stability (such as cholesterol and phosphatidylcholine) and the vectors are being assembled in a different way. A similar change was observed in liposomal formulations of pDNA which were described in section 1.1.6 (106, 107). Recognizing that liposomes which are optimized for delivery of asODN, or pDNA, *in vitro*, are not necessarily the best formulations to use *in vivo*, is an important step in designing suitable carriers.

1.13 Thesis outline

The introduction provides a background to the current state of antisense therapeutics and liposomal drug delivery. Chapters 2, 3, and 4 describe some of the experimental work which was carried out in order to fulfill the objectives and test the hypotheses set out in section 1.2. Chapter 2 characterizes a formulation of asODN which is passively entrapped within the aqueous interior of neutral liposomes (passively entrapped liposomal antisense, PELA), as well as the development of a novel formulation which contains cationic lipid. Neutral liposomes which passively entrap asODN offer several advantages over cationic lipoplexes, such as increased stability, smaller diameter, and possibly a better pharmacokinetic profile. However, one significant draw-back which is highlighted in Chapter 2 is that the loading efficiency of asODN within small diameter (< 200 nm) neutral liposomes quite low. Chapter 2 also describes the development of a novel liposome formulation for asODN, coated cationic liposomes (CCL). Particular attention is given to the physical characteristics such as the diameter and loading efficiency as well as the stability in plasma.

In Chapter 3 the pharmacokinetics and biodistribution of the novel CCL formulation of asODN is evaluated and compared with the PELA formulation. As described earlier, it is important to optimize liposomal asODN formulations for *in vivo* applications; for this reason, the pharmacokinetics and biodistribution were considered before the activity. The literature illustrates quite effectively that *in vitro* activity of free or liposomal asODN does not correlate with the *in vivo* activity.

Chapter 4 attempts to evaluate the activity of the CCL formulation in an *in vitro* model of multidrug-resistance (MDR) in a human B-lymphoma cell line. The MDR phenotype is mediated by P-glycoprotein (P-gp) which is encoded by the *MDR1* gene. The cell line also provided the opportunity to test the effectiveness of antibody-targeted CCL, since it expresses CD19 which internalizes upon binding to anti-CD19 mAb. These cells were first characterized with respect to the MDR phenotype by measuring *MDR1* mRNA expression, P-gp expression and activity, and resistance to doxorubicin which is a substrate for P-gp. The goal was to evaluate the different liposomal formulations in this *in vitro* model, and then move to *in vivo* therapeutic experiments.

A summarizing discussion, with conclusions and future directions is presented in Chapter 5. The results of the project are discussed with reference to the objectives and hypotheses. Conclusions are drawn and suggestions for future experiments are presented.

CHAPTER 2

A new liposomal formulation for antisense oligodeoxynucleotides with small size, high loading efficiency and good stability.

2.1 ABSTRACT

Antisense oligodeoxynucleotides (asODN) are therapeutic agents which are designed to inhibit the expression of disease-related genes. However, their therapeutic use may be hindered due to their rapid clearance from blood and their inefficiency at crossing cell membranes. Cationic liposome complexes have been used to enhance the intracellular delivery of asODN *in vitro*; however, this type of carrier has unfavourable pharmacokinetics for most *in vivo* applications. Significant therapeutic activity of cationic liposomal asODN following systemic administration has not been demonstrated. In an effort to develop improved liposomal carriers for asODN for *in vivo* applications, I have evaluated the physical characteristics of two formulations which represent alternatives to cationic liposome-asODN complexes: asODN passively entrapped within neutral liposomes (PELA) and asODN formulated in a novel coated cationic liposomal formulation (CCL). My results confirm that PELA can be extruded to small diameters which are suitable for iv administration. PELA are stable in human plasma, however, the loading efficiency is relatively low (~ 20%). The CCL formulation can also be extruded to small diameters (< 200 nm), but with significantly higher (80 - 100%) loading efficiency, and they are stable in 50% human plasma at 37°C. A liposomal carrier for asODN with these characteristics may provide a significant therapeutic advantage over free asODN for some therapeutic applications.

2.2 INTRODUCTION

AsODN can be used to decrease the expression of disease-related genes by inhibiting the translation of the mRNA of the target gene, through several possible mechanisms (reviewed in (13)). AsODN must cross the plasma membrane in order to reach their site of action, and liposomes have been widely used to increase the intracellular delivery of asODN.

Different liposomal formulations have been used to deliver asODN to cells; however, the most widely used formulation at present consists of pre-formed cationic liposomes, with or without DOPE, mixed with asODN (94). *In vitro*, these liposomes have been shown to increase the intracellular delivery of asODN, thus increasing the concentration and activity of asODN. The electrostatic interaction between the positively charged lipid and the negatively charged asODN results in the formation of lipid-asODN complexes or lipoplexes. Cationic lipoplexes are usually formulated with excess positive charge in order to mediate interaction with cell membranes which carry a net negative charge. Lipoplexes are very efficient at loading and delivering asODN to cells *in vitro* and have been shown in many systems to be necessary for an antisense activity (94, 126). *In vivo*, the utility of lipoplexes is less clear, and there are no examples which convincingly demonstrate the activity of asODN following i.v. administration. This is because lipoplexes have been optimized for *in vitro* applications, and *in vivo* their large size and excess positive charge result in unfavourable pharmacokinetics, mainly due to rapid uptake into tissues of the mononuclear phagocytic system (MPS).

Since asODN are hydrophilic, they can be passively encapsulated within the aqueous space of non-cationic liposomes, and this method has been used in several published reports (24, 113, 114, 125, 127-129). The main advantage of this type of carrier is that the liposomes can be reduced to small diameters and there is no positive charge to trigger opsonization and MPS uptake. These properties make neutral liposomes attractive candidates for i.v. delivery of hydrophilic drugs like asODN. In addition, polyethylene glycol (PEG) can be grafted onto the liposome surface which decreases the uptake of the liposomes into the MPS, leading to increased circulation times and enhanced localization of liposomal contents in diseased tissues (66, 86, 88, 130, 131).

An important limitation to passive encapsulation of asODN within the aqueous space of liposomes is the poor loading efficiency, especially within small (< 200 nm) liposomes. For example, hydration of a dried film of neutral lipids with a concentrated solution of a 15-mer phosphodiester asODN resulted in only 3% loading following extrusion through a 200 nm unipore filter (113). In another study, liposomes encapsulating a 15-mer asODN were prepared using the reverse phase evaporation method and 10% loading efficiency was reported into liposomes 170 nm in diameter (128). Other studies fail to present details describing the loading efficiency, the asODN to lipid ratio, or liposome size (24, 125), and these are all important parameters to consider in the development and use of a liposomal carrier for asODN. Even when novel protocols are developed which increase the encapsulation efficiencies of oligonucleotides to 50-70%, the liposome size is not reported (114, 132). An additional criticism is that most of these studies made no effort to distinguish entrapped asODN from that passively associated with the outside of the liposomal membrane.

The purpose of the present study was two-fold: first, I wanted to determine the encapsulation efficiency of asODN within the aqueous space of small (<200 nm) neutral liposomes containing PEG-DSPE. My results indicate that the addition of 2-5 mol% PEG-DSPE into neutral liposomes approximately doubles the loading efficiency of asODN from less than 10% to 20%. The second purpose of the study was to develop and characterise a liposomal carrier for asODN containing cationic lipid, which would be suitable for i.v. administration. By using a method which optimises the charge interaction between the asODN and the cationic lipid, combined with a method which provides an outer coating of neutral lipid, we have been able to produce a liposomal carrier which is very efficient at loading asODN (80 - 100%), has a small diameter (<200 nm), and is stable in human plasma. The present manuscript provides a detailed description of PEG-containing liposomes passively entrapping asODN (PELA) and a novel formulation containing cationic lipid-asODN particles coated with PEG-DSPE and neutral lipids (CCL).

2.3 MATERIALS AND METHODS

2.3.1 Materials

Partially hydrogenated egg phosphatidylcholine iodine number 40 (PC40) and PEG (molecular weight 2000) covalently attached to distearoylphosphatidylethanolamine (PEG-DSPE) were generous gifts from SEQUUS Pharmaceuticals, Inc. (Menlo Park, CA) and have been described elsewhere (133, 134). Cholesterol (CHOL) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL). Sepharose CL-4B, Na-^{125}I (560-625 Mbq/μg iodine), and scintillation fluor/aqueous counting scintillant were purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Iodogen was purchased from Pierce Chemical Co. (Rockford, IL). Bio-Spin 6 chromatography columns were purchased from Bio-Rad (Hercules, CA). Metrizamide (Grade 1) was purchased from Sigma Chemical Co. (St. Louis, MO). Cholesteryl-[1,2-^{3}H-(N)]-hexadecyl ether (^{3}H-CHE), 1.48-1.22 Tbq/mmol, was purchased from New England Nuclear (Mississauga, ON). Adenosine-5'-triphosphate, [γ-^{32}P] (γ-^{32}P-ATP) (110-167 Tbq/mmol) was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA) or the Department of Biochemistry DNA Core Services Laboratory at the University of Alberta (Edmonton, AB). T4 polynucleotide kinase was purchased from Gibco BRL (Burlington, ON). Phosphorothioate ODN (18-mers: scrambled or complementary to the initiation codon of the *MDR1* gene) were synthesized by the University Core DNA Services Lab at the University of Calgary (Calgary, AB). PEI-cellulose chromatography sheets were purchased from J.T. Baker, Inc. (Phillipsburg, NJ). Nuclepore® polycarbonate filters for extrusion were purchased from Corning Costar (Kennebunk, ME). All other chemicals were of analytical grade quality.

2.3.2 Preparation of radiolabelled oligodeoxynucleotides

^{32}P-ODN and ^{125}I-ODN were prepared and used as tracers in order to follow the loading of ODN within the different liposomal formulations. Phosphorothioate ODNs were labelled with ^{125}I using Iodogen similar to the method described by Piatyszek *et al.* (135). A 1 ml reaction vial was coated with a thin film of Iodogen by dissolving approximately 1 mg of Iodogen in chloroform and then drying the solution under a stream of nitrogen. Next, 100-500 μg ODN in 50 μl ddH$_2$O along with 300 μl of 0.35 M sodium acetate pH 4.0 and 185 Mbq (2,300 pmol) of ^{125}I in a volume of 50 μl were added to the vial and incubated at 40°C for 45 minutes. Free ^{125}I was removed on a Sephadex G-15 column. Dialysis or precipitation of the ODN, followed by rehydration, resulted in less than 5% free ^{125}I. The specific activity of the ^{125}I-ODN was approximately 4 Mbq/nmol. This protocol results in the ^{125}I label being covalently attached at the C-5 position of cytosine nucleobases (135, 136).

In some experiments, ^{32}P-ODNs were also used in order to determine loading efficiency. T4 polynucleotide kinase and forward labelling buffer were used as outlined by the manufacturer. Free γ-^{32}P-ATP was separated using a Bio-Spin 6 column. The specific activity of labelled phosphorothioate ODNs was approximately 9 Mbq/nmol. Contamination of the sample by γ-^{32}P-ATP or free $^{32}PO_4^{-3}$ was negligible as determined by thin layer chromatography.

2.3.3 Preparation of liposomes passively entrapping ODN

Passively entrapped liposomal ODN (PELA) were prepared by a method similar to that described by Thierry *et al.* (114, 132). PC40:CHOL:PEG-DSPE were mixed at a 2:1:0, 2:1:0.04, 2:1:0.08 or 2:1:0.1 molar ratio (3 μmol total phospholipid) in $CHCl_3$ (along with a trace of ^{3}H-CHE) and dried to a thin film by rotary evaporation. Nine μl of a 10 mg/ml solution of ODN in distilled deionized water (ddH_20), plus trace ^{125}I-ODN, was added and the film was hydrated overnight at 4°C. The following day an additional 9 μl of 10 mg/ml ODN (including trace ^{125}I-ODN) was added and the tube was vortexed vigorously for 30 seconds. Next, 18 μl of HEPES buffer (25 mM HEPES, 140 mM NaCl, pH 7.4) was added and the sample was vortexed vigorously. After a two hour incubation at room temperature the tubes were sonicated for three minutes in a bath sonicator. An additional 450 μl of HEPES buffer was added and the liposomes were extruded with a syringe-tip extruder through progressively smaller polycarbonate filters (800 nm to 200 nm). In some experiments, prior to the extrusion step, liposomes were subjected to ten freeze (liquid N_2) and thaw (40°C) cycles following sonication. Liposome size was determined by dynamic light scattering using a Brookhaven B190 submicron particle analyser (Brookhaven Instruments Corp., Holtsville, NY). Free ODN was separated from encapsulated ODN by passing the mixture down a Sepharose CL-4B column (1 x 20 cm).

2.3.4 Preparation of coated cationic liposomes

Bligh and Dyer extractions (137), using the cationic lipid DOTAP, and an 18-mer phosphorothioate ODN, were carried out in a similar method to that used for plasmid DNA (110, 111). DOTAP was diluted in 0.25 ml $CHCl_3$, and 0.52 ml of CH_3OH was added followed by 0.25 ml of ODN diluted in ddH_20 (unless otherwise specified). Following 30 minutes at room temperature, 0.25 ml of $CHCl_3$ and 0.25 ml of ddH_2O were added and the tubes were centrifuged for seven minutes at 830 x g. Following centrifugation, the phases were separated and radioactivity measured, or the absorbance at 260 nm (A_{260}) was measured in the aqueous phase, to determine the amount of ODN extracted.

Following extraction and removal of the aqueous phase, PC40, CHOL, and PEG-DSPE were added to the organic phase at the indicated ratios. ddH_2O was then added (to give 10 to 30 mM lipid concentration in the water volume) and the tube was vortexed vigorously (20 seconds) and then sonicated for one minute to produce a stable emulsion. $CHCl_3$ was then evaporated under vacuum (~ 500 mmHg) on a rotary evaporator until a gel phase was reached. Subsequent evaporation lead to the inversion of the system from a gel to a liquid. I hypothesize that during this procedure the DOTAP-ODN particles are coated with non-cationic lipids. The following experiments describe the characterization of coated cationic liposomes (CCL) prepared in this way.

Some CCL formulations (those lacking PEG-DSPE) would not migrate on a Sepharose CL-4B column, possibly due to aggregation, and therefore loading efficiency was determined by separation on a discontinuous metrizamide gradient. Twenty and 10% metrizamide were prepared in ddH_2O and gradients were set up with 20% in the bottom of the tube (2.5 ml), 10% in the middle (7 ml) and 0% (2.5 ml) on top. Liposomes were mixed with the 20% layer prior to set-up and the tubes were centrifuged for 6 - 12 hours at approximately 200,000 x g. Liposomes localized primarily at the 10%/0% interface.

2.4 Results

2.4.1 Effect of PEG-DSPE on passive entrapment of ODN

The first set of experiments was performed in order to determine the effect of PEG-DSPE on the loading efficiency of ODN within the aqueous space of neutral liposomes (PELA). Passive loading has been used in the past to entrap DNA or ODN in liposomes, however, the size of liposomes produced and the loading efficiency are not often described in detail. Therefore, an effort was made to describe accurately the conditions, the final diameter, the loading efficiency, the ODN/lipid ratio and also the trapped volume of liposomes produced.

The effect of PEG-DSPE on ODN entrapment efficiency was examined for

PC40:CHOL liposomes of approximately 200 nm in diameter (Table 2.1). In order to determine the percentage of ODN which may be associated with the liposome exterior, liposomes were made by the method described, except ODN was not included in the hydration buffer. ODN was added to the liposomal suspension just before extrusion and then the mixture was separated on a Sepharose CL-4B column. Less than 1% of the added ODN eluted with the lipid for all three groups (0, 2, 4 mol% PEG-DSPE) (data not shown). Therefore, values for loading efficiency presented in Table 2.1 can be taken to represent ODN mainly encapsulated within the aqueous space of liposomes.

The results in Table 2.1 indicate that by adding 2 or 4 mol% PEG-DSPE to PC40:CHOL liposomes, the trapping efficiency of ODN approximately doubled compared to liposomes lacking PEG-DSPE (not significantly significant P>0.05). The loading data are also expressed as ODN to phospholipid ratios and as trapped volumes to facilitate comparisons with other published data. The trapped volumes were calculated using radiolabelled ODN as the aqueous phase solute, and the results are consistent with expected values for liposomes of this size, prepared in this way, and suggests that the liposomes lacking PEG-DSPE are multilamellar (138). Subjecting the liposomes to freezing and thawing did not increase the loading efficiency of ODN within the aqueous space of these liposomes.

Table 2.1: Encapsulation of oligodeoxynucleotides within neutral liposomes containing increasing concentrations of PEG-DSPE. Each value represents the mean of three experiments ± standard deviation.

mol % PEG-DSPE	**Size (nm)**	**Trapped volume (μl/μmol PL)**	**Loading efficiency (%)**	**nmol asODN/ μmol PL**
0	230 ± 10	1.0 ± 0.35	8 ± 3.2	0.6 ± 0.35
2	190 ± 12	2.5 ± 0.84	15 ± 7.7	1.6 ± 0.80
4	190 ± 15	2.3 ± 0.30	19 ± 3.6	1.4 ± 0.36

2.4.2 Formation of hydrophobic ODN/DOTAP particles

Reimer *et al.* described the formation of hydrophobic plasmid DNA-cationic lipid particles through an organic extraction procedure (110). They demonstrated that cationic lipids could be used to extract plasmid DNA from an aqueous phase, into an organic phase, through a Bligh and Dyer monophase (137). The extraction was shown to be mediated by the electrostatic interaction between the positively charged lipid and the negatively charged DNA. This procedure should also be applicable to negatively charged asODN, and I hypothesized that the hydrophobic particles in the organic phase could be coated with neutral lipids through a reverse evaporation step. I predicted that this procedure would result in liposomes having a high trapping efficiency for asODN. Furthermore, the addition of neutral lipids, including PEG-DSPE, would serve to coat the cationic lipid-ODN particles, adding stability, decreasing the non-specific adsorption of plasma proteins and increasing their circulation half-lives.

The first step was to evaluate whether the extraction procedure is applicable to asODN, and to determine the amount of cationic lipid required for efficient extraction of the asODN into the organic phase. Ten µg of 18-mer phosphorothioate asODN (including trace ^{32}P-asODN) was extracted using 0-160 nmol DOTAP through a Bligh and Dyer monophase as described in the Methods section. Figure 2.1 illustrates the results from such an experiment and demonstrates that increasing the amount of DOTAP increases the asODN extracted from the aqueous phase into the organic phase. When 40 nmol or more of DOTAP was used, almost 100% of the asODN could be extracted (Figure 2.1A). These data can be expressed in terms of the +/- charge ratio of DOTAP to asODN phosphate as shown in Figure 2.1B. Thirty nmol DOTAP corresponds approximately to a 1:1 +/- charge ratio with 10 µg asODN and at this ratio approximately 90% of the asODN was extracted into the organic phase. When several replicates of the extraction were done at a 1:1 charge ratio, 90-95% of the asODN was extracted into the organic phase.

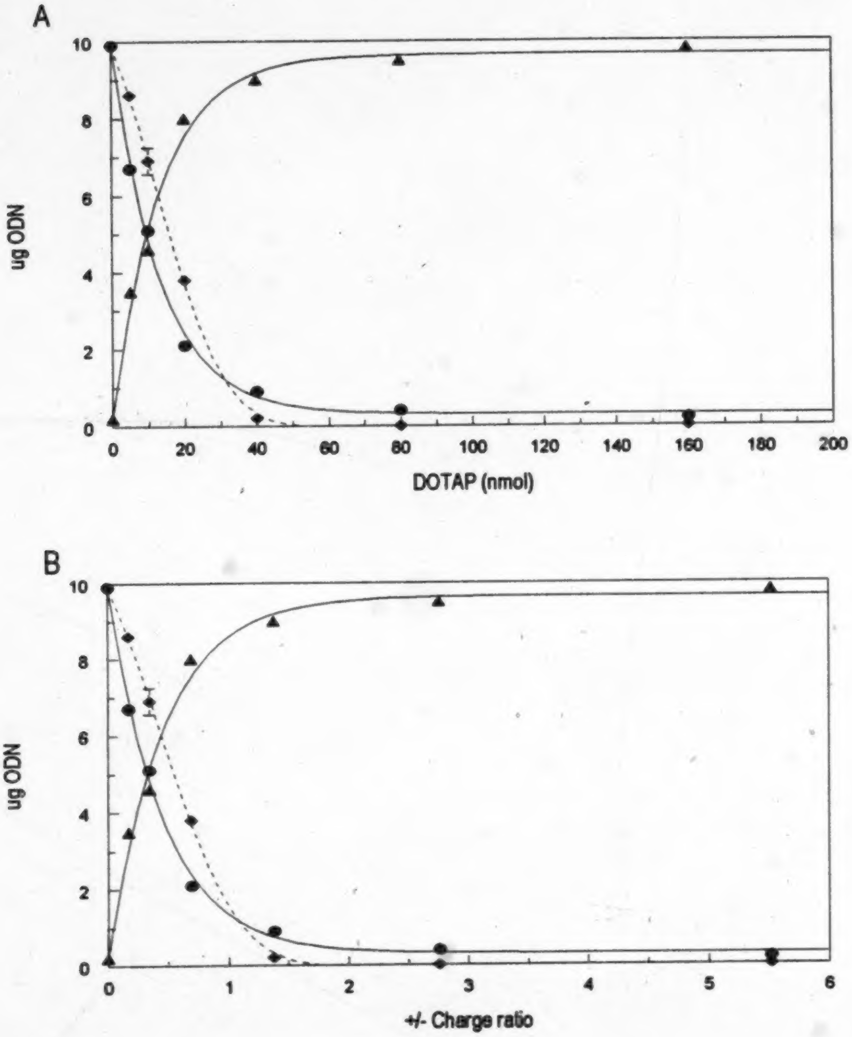

Figure 2.1: Bligh and Dyer extractions of 10 µg 18-mer phosphorothioate asODN using the cationic lipid DOTAP. Triangles represent asODN in the organic phase and circles represent asODN remaining in the aqueous phase as determined by using a radiotracer (^{32}P-asODN). Diamonds represent asODN remaining in the aqueous phase as determined by spectrophotometric assay (mean of 3 experiments ± standard deviation). A) DOTAP expressed as nmol. B) DOTAP expressed as + / - charge ratio.

The assumption in this extraction procedure is that it is the electrostatic interaction between the positively charged DOTAP and the negatively charged asODN which drives the extraction. This is supported by the work of Reimer *et al.* (110), and experiments performed in our laboratory indicate that neutral lipids do not result in the extraction of asODN (data not shown). Experiments have also indicated that the aqueous phase in which the asODN is dissolved plays an important role in the extraction efficiency. Figure 2.2 illustrates the results from a series of extractions carried out using 60 µg asODN and 0.15 µmol DOTAP with the asODN diluted in ddH_2O, 10% sucrose, or 25 mM HEPES buffer containing 140 mM NaCl (pH 7.4). At the DOTAP/asODN ratio in this experiment (+/- = 0.88), more than 80% of the asODN is extracted from the ddH_2O aqueous phase. When the asODN was dissolved in 10% sucrose approximately 70% of the asODN was extracted from the aqueous phase, and the extraction was completely inhibited when 25 mM HEPES, 140 mM NaCl (pH 7.4) was used as the aqueous phase. This is likely caused by a charge-shielding effect of the Na^+ and Cl^- ions preventing the efficient interaction between the DOTAP and the asODN, since a non-ionic solute such as sucrose had less of an effect on the extraction efficiency.

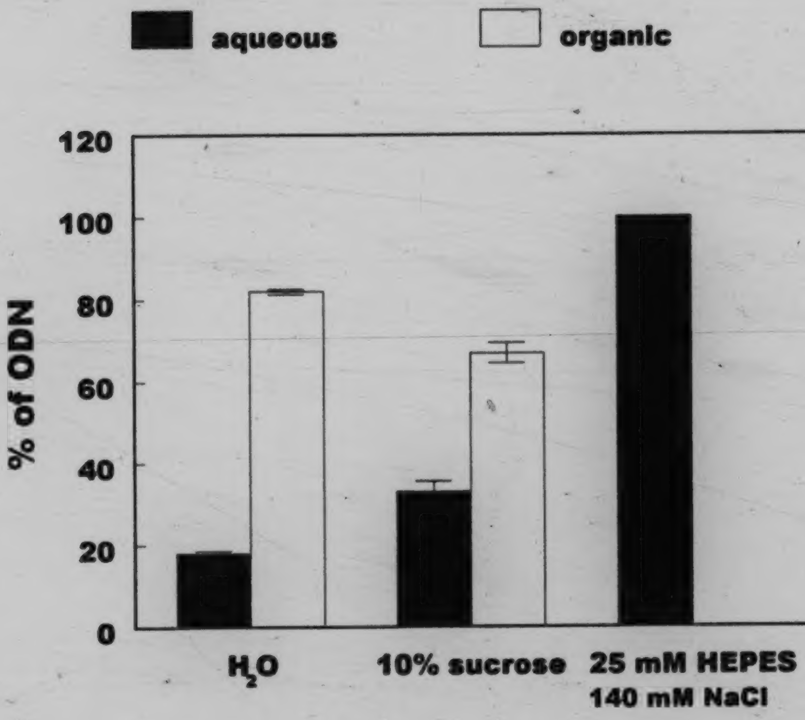

Figure 2.2: Extraction by DOTAP (0.15 μmol) of 60 μg 18-mer phoshorothioate asODN dissolved in different aqueous phases (0.88 +/- charge ratio). Extraction was determined by spectrophotometric assay of the aqueous phase. 100% asODN in the aqueous phase was determined by carrying out the extraction in the absence of DOTAP. Values for asODN in the organic phase were determined as the difference between asODN in the aqueous phase and total asODN added (100%). Each value represents the mean of three experiments ± standard deviation.

2.4.3 Coated cationic liposome formation by reverse phase evaporation

The size and loading efficiency were determined for CCL at different lipid ratios (Table 2.2). Most CCL preparations were formed by extraction of asODN into the organic phase at a 3.5:1 +/- charge ratio which ensured that nearly 100% of the asODN would be extracted into the organic phase. Following extraction of 50 μg asODN with 0.5 μmol DOTAP, the organic phase was isolated and different amounts of coating lipid were added (Table 2.2). ddH_2O was then added and the reverse evaporation was carried out as described. After reversion into the aqueous phase, no visible aggregates or precipitates were present and particle diameter was in the 400-500 nm range. Loading efficiencies were in excess of 70%. The amount of coating lipid had no effect on the loading efficiency, and very little effect on the liposome diameter, for non-extruded liposomes (Table 2.2). Changes in the asODN:PL ratios in Table 2.2 simply reflect differences in the amount of added coating lipid.

For many *in vivo* applications smaller diameter liposomes would be advantageous, so the CCL were extruded to reduce their size. I observed that the amount of coating lipid added affected my ability to extrude CCL. When the PC40:DOTAP molar ratio was less than 4:1, extrusion through 200 nm filters lead to immediate aggregation and destabilization (data not shown). However, at a PC40:DOTAP molar ratio of 3:1, CCL could be extruded through 200 nm filters when 5 mol% PEG-DSPE was included in the coating lipids to prevent aggregation (Table 2.2). Following extrusion through 200 nm

Table 2.2: Size and loading efficiency of liposomes formed by the extraction-reverse evaporation procedure. Each value represents the average ± standard deviation of three experiments, except for the asODN:PL ratio for the last row, which is the result from one experiment.

PC40/CHOL/ DOTAP/PEG-DSPE (μmol)	+/- charge ratio	Size (nm)	Loading efficiency (%)	nmol ODN/ μmol PL
Not extruded				
0.5 / 0.5 / 0.5 / 0	3.5	360 ± 60	72 ± 10	7.9 ± 2.1
1.0 / 0.75 / 0.5 / 0	3.5	530 ± 170	74 ± 4	5.4 ± 0.4
2.0 / 1.25 / 0.5 / 0	3.5	510 ± 200	73 ± 10	3.4 ± 0.5
Extruded				
3.0 / 1.75 / 0.5 / 0.175	3.5	173 ± 4.2	86 ± 5	2.1 ± 0.1
3 / 2 / 1 / 0.2	1	219 ± 28	90 ± 9	17*

* this data is from only one experiment

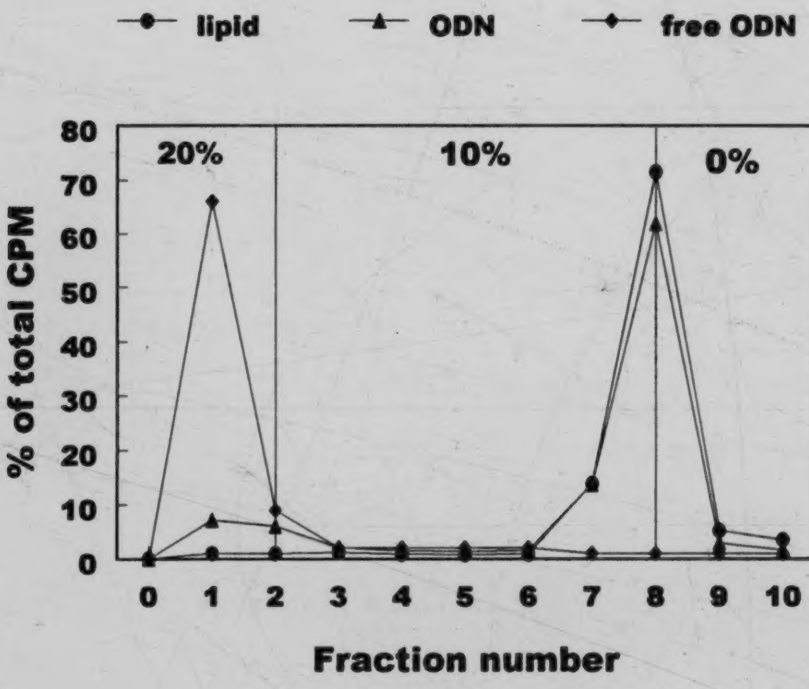

Figure 2.3: Metrizamide gradient profile for CCL containing ^{3}H-CHE as a lipid marker and ^{125}I-asODN as a tracer. CCL were composed of PC40:CHOL:DOTAP:PEG-DSPE (3.0 / 1.75 / 0.5 / 0.175 µmol). Gradients were fractionated by making a small hole in the bottom of the plastic tube and collecting fractions of equal drops. The first 2 fractions contained the majority of the 20% metrizamide layer, while fractions 3-8 contained the majority of the 10% layer. Closed circles (●) represent lipid and closed triangles (▲) represent asODN formulated in CCL. Diamonds (◆) represent a control showing the lack of migration of free asODN on the gradient.

polycarbonate filters, the average diameter of CCL was 173 nm (Table 2.2). Loading efficiencies were determined on a discontinuous metrizamide gradient (Figure 2.3). Free ^{125}I-asODN remained at the bottom of the gradient (20% metrizamide), while liposomes, along with the loaded asODN, migrated to the 10%/0% interface. For the experiment represented in Figure 2.3, approximately 86% of the asODN migrated through the gradient with the lipid fraction and the average of three experiments are presented in Table 2.2.

Separation on a metrizamide gradient does not discriminate between spuriously associated asODN on the liposome exterior and that in the aqueous liposome interior. Therefore the amount of asODN which could associate with the liposome exterior was determined by making empty (without asODN) CCL by the procedure outlined above, and then adding asODN before separation on metrizamide gradients. When formulated in this manner, approximately 50% of the asODN bound to the liposome exterior and migrated through the gradient with the lipid. However, when the liposomes are made in the absence of asODN, a significant amount of DOTAP (i.e., positive charge) may be present in the outer phospholipid monolayer of the liposome and available for binding to asODN added to the liposome exterior. When CCLs are made from extracted DOTAP/asODN particles, I propose that the DOTAP is sequestered primarily in the liposome interior, associated with asODN.

For *in vivo* applications, a carrier system lacking excess positive charge would be desirable to reduce MPS uptake. At a 1:1 +/- charge ratio of DOTAP to asODN phosphate, 90-95% of asODN was extracted into the organic phase, however, when neutral lipids were added without PEG-DSPE, lipid aggregates formed during the reverse evaporation procedure. It has been observed in other systems that significant aggregation occurs as the +/- charge ratio approaches one (139) and addition of PEG has been shown to prevent aggregation and precipitation in these electrostatic systems (42). In the presence of 5 mol% PEG-DSPE no aggregation was observed and sequential extrusion through 200 nm filters resulted in an average particle diameter of 219 nm and a loading efficiency of 90% (Table 2.2).

2.4.5 Effect of plasma on stability and dissociation of asODN

The stability of CCL and PELA in plasma-containing medium at 37°C was evaluated. CCL and PELA were prepared at a 1:1 charge ratio DOTAP:asODN, and contained 5 mol% PEG-DSPE. PELA had an average diameter of 185 ± 4 nm and a 21% trapping efficiency while the CCL had an average diameter of 190 ± 2 nm and an 80% trapping efficiency. An aliquot from each group was diluted in an equal volume of human plasma and the samples were incubated at 37°C for 24 hours. No increase in diameter was observed for either preparation. Following fractionation of each preparation on Sepharose CL-4B columns, the asODN eluted almost exclusively with the liposomes in the void volume (Figure 2.4). These results demonstrate that both formulations were able to maintain their size and stability with minimal leakage of asODN in plasma-containing media.

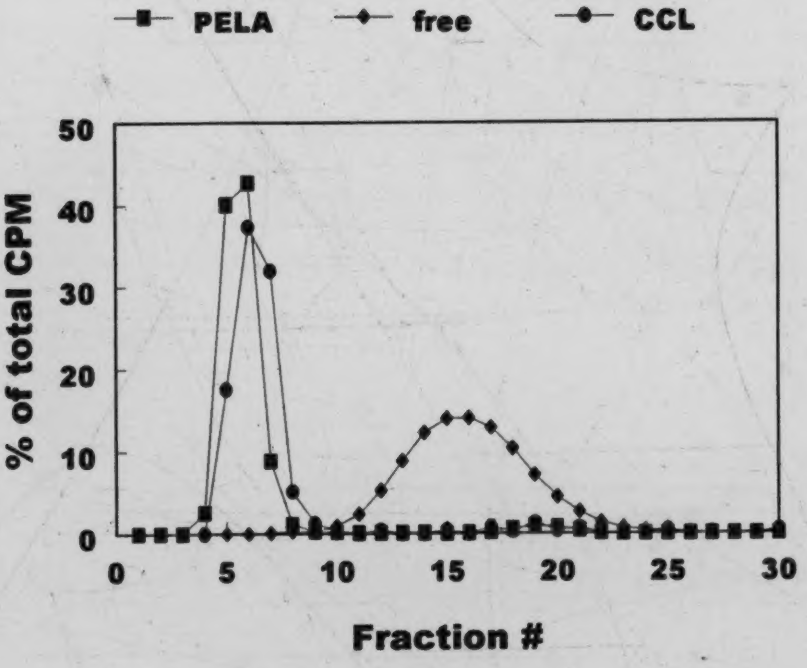

Figure 2.4: Sepharose CL-4B column profile of PELA and CCL incubated at 37°C for 24 hours in 50% human plasma. ^{32}P-asODN was used as a tracer. Fractions 4 to 8 represent the lipid fractions as determined by visual observation and by using ^{3}H-CHE as a lipid tracer (counts not shown). Closed squares represent asODN passively entrapped within PC40:CHOL (2:1 molar) liposomes containing 5 mol% PEG-DSPE, while closed circles represent asODN formulated in CCL with the composition PC40:CHOL:DOTAP:PEG-DSPE (3:2:1:0.2 molar). Closed diamonds represent free asODN (including ^{32}P-asODN tracer) incubated with 50% human plasma.

2.5 DISCUSSION

In this study we examined the physical characteristics (diameter, stability) and loading efficiency of two different liposomal formulations of asODN which may be suitable for *in vivo* applications. Cationic lipoplexes are not suitable for systemic administration due to their large size and excess positive charge which leads to their rapid removal from circulation, primarily into liver and lung. Pharmacokinetic studies have demonstrated that cationic lipoplexes are unable to deliver asODN to tissues other than the MPS and lung (120, 121).

Passive encapsulation within neutral liposomes is an alternative to using cationic lipoplexes; however, my results confirm that this is an inefficient process and only a fraction of the added asODN is loaded. An unexpected finding was that the loading efficiency of an 18-mer phosphorothioate asODN was increased by the addition of 2-5 mol% PEG-DSPE. This may be a result of an increased trapped volume due to decreased lamellarity of the liposomes and, while it is an interesting observation, the loading efficiency obtained was still quite low (20%).

It is difficult to compare the loading efficiencies observed in my study with previously published results. As mentioned in the Introduction, few studies give sufficient details to characterize properly the encapsulation of asODN within neutral liposomes. For example, Thierry *et al.* reported loading efficiencies of 50-70% of added asODN. However, the size of the liposomes was not reported, and small size is an important consideration for *in vivo* applications (114, 132).

Neutral PEG-containing liposomes have longer circulation half-lives *in vivo* than cationic lipoplexes lacking PEG (66, 120, 121, 134, 140). However, as described above, these liposomes have poor loading efficiencies. Therefore, I developed a CCL formulation which combines the benefits of neutral liposomes, such as stability and long circulations times, with the high loading efficiency of asODN obtained with cationic lipoplexes. CCL are produced in a two step process in which asODN are first complexed with cationic lipid at a 1:1 charge ratio, followed by a coating procedure in which neutral lipids (with or without PEG-DSPE) are added to produce the coating layer. CCL can be extruded to diameters below 200 nm, they are stable in plasma and have trapping efficiencies of around 90%. My results indicate that asODN can be extracted through a Bligh and Dyer monophase into an organic phase using cationic lipid, similar to the extraction of plasmid DNA as described by Reimer *et al.* (110) and I have demonstrated that the cationic lipid-asODN hydrophobic complexes serve as useful intermediates in the formation of CCL through a reverse evaporation procedure.

The exact structure of the hydrophobic particles produced by the extraction procedure is unknown, but I can make a prediction (Figure 2.5). In order for the asODN to exist in a hydrophobic environment such as $CHCl_3$ it must be shielded by the lipid, possibly in the form of an inverted micelle (Figure 2.5C). This structure serves as a useful intermediate in the formation of liposomes through a reverse phase evaporation method

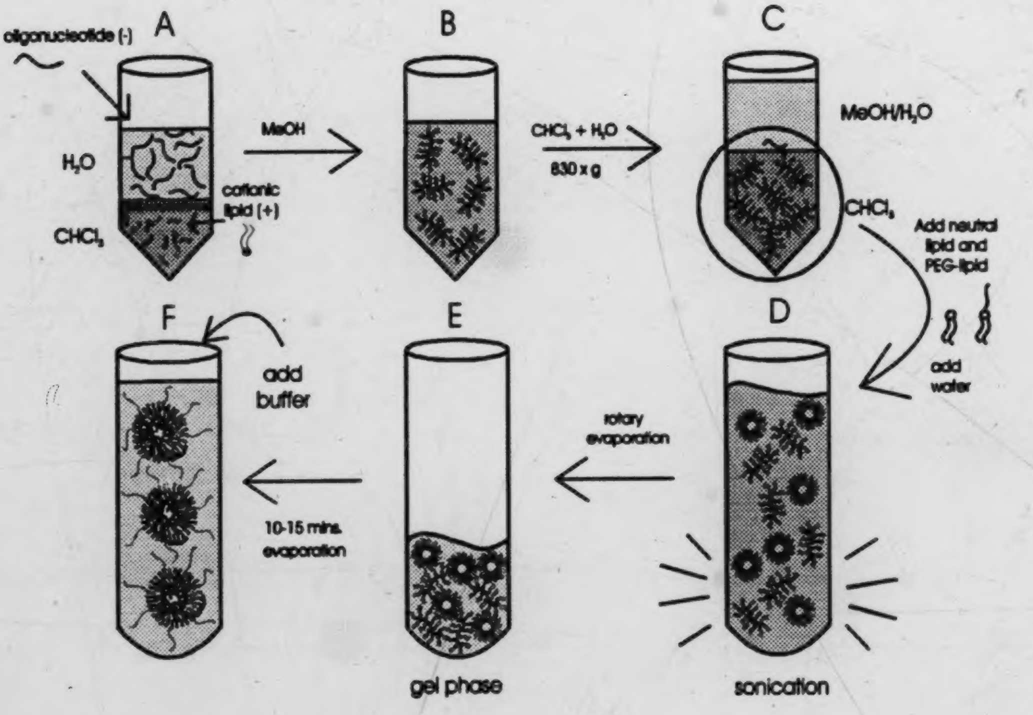
oligonucleotide (-)
A
B
C
H_2O
$CHCl_3$
cationic lipid (+)
MeOH
$CHCl_3 + H_2O$
830 x g
$MeOH/H_2O$
$CHCl_3$
Add neutral lipid and PEG-lipid
add water
D
E
F
add buffer
rotary evaporation
10-15 mins. evaporation
gel phase
sonication

Figure 2.5: Schematic demonstrating the steps and proposed intermediate structures involved in making coated cationic liposomes (CCL).

similar to that described by Szoka and Papahadjopoulos (119). The outline for such a procedure is described in Figure 2.5C-F. By formulating the asODN and cationic lipid in this way, I am attempting to sequester a majority of the cationic lipids and the asODN within a coating of neutral and PEG-modified lipids. In this way, the cationic lipid serves to load efficiently the asODN, but is not available for interaction with cells or plasma proteins. This model for CCL formation is supported by my results demonstrating a lack of aggregation or release of asODN in the presence of human plasma (Fig. 4). Similarly, PELA did not aggregate or leak, however, this is not surprising since these liposomes contain 5 mol% PEG-DSPE and no cationic lipid.

Very recently, other formulations have been developed which attempt to incorporate some of these characteristics into a liposomal carrier for asODN, for example, the LPDII particles described by Li *et al.* (105). LPDII particles are 150-200 nm in diameter and entrap approximately 60% of added asODN; however, their stability or activity in the presence of plasma have not been demonstrated. Meyer *et al.* mixed asODN with pre-formed liposomes composed of DOPE, PEG-PE and a cationic lipid (141). The authors report very good loading with a high asODN/lipid ratio, and suggest that the complexes are stable in plasma; however, roughly 40% of the asODN dissociated from liposomes upon incubation in 50% plasma, and pharmacokinetic experiments indicated that even more dissociation occurs upon i.v. injection (D. Kirpotin personal communication). This is probably a result of asODN associated with the outside of the liposomes which dissociates in plasma. My experiments indicate that up to 50% of asODN added to the outside of CCL, formed in the absence of asODN, would bind to the CCL and could dissociate. However, when CCL were formed with asODN present during the extraction I observed no dissociation of the asODN in plasma suggesting that the asODN is sequestered in the interior of the liposomes in association with the cationic lipid (Figure 2.5F).

In the CCL formulation I used a charge neutral ratio of DOTAP:asODN and added 5 mol% PEG-DSPE in order to prevent aggregation and decrease adsorption of serum proteins and interaction with non-target cells. This is in contrast to typical cationic lipid-asODN complexes which carry an excess positive charge and results in non-specific interactions with most cell types. Experiments in our laboratory indicate that cells treated with LipofectAMINE®-asODN complexes take up more asODN than cells treated with asODN formulated in CCL (142). However, when CCL are targeted by coupling a mAb to the surface, the levels of cell-associated asODN are increased significantly, and approach levels observed following treatment delivery using LipofectAMINE®. In a mixed cell population, for example *in vivo*, it would be an advantage to use an asODN carrier which has selectivity for the target cell population, rather than one which interacts non-specifically (albeit efficiently) with all cell types encountered.

Over the past decade, it has become apparent that liposomes which are able to circulate in the bloodstream, without rapid uptake into the liver or spleen, may be able to passively target sites of disease such as solid tumours and sites of infection (64, 130, 143-147). A separate manuscript (submitted for publication) demonstrates that the CCL formulation has long-circulating pharmacokinetics similar to PELA and therefore may be able to take advantage of this passive targeting effect. Other studies have demonstrated that enhanced circulation times are required for ligand-mediated targeting of liposomes (85) and this targeting has been shown to result in enhanced antitumour activity when the liposomes are loaded with doxorubicin (86, 88). The CCL formulation can also be targeted to a specific cell type by adding a coupling lipid (e.g. 4-(p-maleimidophenyl) butyrate - PEG-DSPE) to the coating lipids and attaching a targeting ligand following CCL formation. Another paper (submitted for publication) demonstrates that antibody-targeted CCL (and to a lesser extent non-targeted CCL) are effective at delivering a c-myb asODN resulting in an antiproliferative effect in a neuroblastoma cell line.

Significant progress has been made in recent years in bringing asODN into the clinical setting, and there are several early to mid-phase clinical trials using asODN to treat diseases ranging from cancer to human immunodeficiency virus (HIV) infection to inflammatory conditions. However, only one asODN has received approval for marketing in the United States, and that is a phosphorothioate anti-cytomegalovirus-asODN used to treat CMV retinitis in AIDS patients (22). The anti-CMV-asODN is administered via local (intravitreal) injection and is not subject to significant redistribution via the bloodstream. However, the i.v. route of administration will be most convenient for asODN targeted to other diseases such as cancer or HIV infection and, in these situations, the activity of the asODN may be significantly improved by the use of a well-designed liposomal carrier. The CCL formulation which I have described meets many of the requirements of a useful drug carrier and could significantly increase the therapeutic activity of asODN.

A novel, long-circulating and functional liposomal formulation of antisense oligodeoxynucleotides targeted against *MDR1*.

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3.1 ABSTRACT

The goal of this study was to develop a small, stable liposomal carrier for antisense oligonucleotides (asODN) which would have high trapping efficiencies and long circulation times *in vivo*. Traditional cationic liposomes aggregate to large complexes and when injected i.v., rapidly accumulate in the liver and lung. I produced charge-neutralized liposome-asODN particles by optimizing the charge interaction between a cationic lipid and negatively charged asODN, followed by a procedure in which a layer of neutral lipids coated the exterior of the cationic lipid-asODN particle. The coated cationic liposomes (CCL) had an average diameter of 188 nm and entrapped 85-95% of the asODN. The biodistribution and pharmacokinetics of an 18-mer ^{125}I-labelled phosphorothioate ODN formulated by this method was determined following tail vein injection in mice. The majority of the asODN was cleared from blood with a half-life of over 10 hours compared to less than 1 hour for free asODN. A number of therapeutic opportunities exist for the use of small, stable, long-circulating and targetable liposomal carriers such as this, with high trapping efficiencies for asODN.

3.2 INTRODUCTION

Antisense oligodeoxynucleotides (asODNs) comprise a group of therapeutic agents specific for the treatment of diseases which occur as the result of the overexpression of a normal gene, the expression of a mutant gene, or the presence of an infectious agent. Several oncogenes or cancer-related genes have been examined as potential targets for modulation by asODN (reviewed in (25)). Down-regulation of gene expression has been demonstrated in many *in vitro* systems (reviewed in (18)) and there are also published reports of asODN inhibition of target gene expression in animal models of cancer (148-150), cardiovascular disease (151) and several central nervous system applications (152-154). There are currently at least 12 clinical trials using asODNs to treat diseases ranging from Crohn's disease, to Non-Hodgkin's lymphoma, and to viral infections such as human immunodeficiency virus and cytomegalovirus (21). Throughout this period of development, liposomes have played an important role as carriers for asODNs.

In vitro, cationic liposomes have been shown to enhance dramatically the uptake and pharmacological activity of asODNs and, in some instances, cationic liposomes may be absolutely necessary for the activity of asODNs (94, 114, 155, 156). Commonly used formulations consist of cationic lipids mixed in approximately equimolar concentrations with the helper lipid DOPE, similar to the transfection complexes used to deliver plasmid DNA (157-159). Preformed cationic liposomes are mixed with asODNs, and cationic lipid-asODN complexes are formed through the electrostatic interactions. These complexes are very effective at entrapping asODNs and mediating their delivery to cells *in vitro*.

In vivo, the requirement for a lipid carrier is much less clear. Several examples of the therapeutic activity of asODNs in animal models as well as clinical trials in humans exist, and in these examples lipid carriers have not been used. However, free asODNs are rapidly cleared from circulation and improvements in the pharmacokinetics and specific activity of asODNs may be possible with the use of a well-designed lipid carrier. Understanding the pharmacokinetics and biodistribution of liposomal asODN is an important prelude to designing appropriate clinical applications. Two previous studies on the biodistribution of cationic liposomal phosphorothioate asODN complexes found that, immediately following i.v. injection, the complexes accumulated primarily in lung and liver (120, 121). The liposome formulations were similar to those used *in vitro*, a cationic lipid mixed with DOPE and then complexed with asODNs. However the relatively static environment of a tissue culture dish is quite unlike the *in vivo* environment, and therefore some effort should be made to optimize liposomal asODN carriers if they are to be used *in vivo*.

Past studies using liposomes as drug carriers *in vivo* have shown that liposomes must be able to avoid uptake into the liver and spleen in order to achieve passive, or ligand mediated, targeting (82, 85). The extremely short circulation times of the cationic liposome-asODN complexes described previously would hinder targeting to diseased tissues *in vivo*. In addition, interaction of these complexes with plasma proteins and non-target cells as a result of the excess positive charge that these complexes carry, would interfere with ligand-mediated targeting to a specific cell type.

In this study, my goal was to develop a liposomal carrier which would be efficient at entrapping asODNs, be stable and of small (< 200 nm) size, and be able to circulate in blood for extended periods following i.v. administration. I have adapted a procedure (110) described by Reimer *et al.* which facilitates the electrostatic interaction between a cationic lipid and plasmid DNA and results in hydrophobic complexes following extraction through a Bligh and Dyer monophase (137). The extraction can also be used for phosphorothioate asODN. I hypothesized that the hydrophobic cationic lipid-asODN particles could be coated with neutral phospholipids and lipid derivatives of PEG through a reverse phase evaporation procedure similar to that described by Szoka and Papahadjopoulos (119). Sequestration of positive charges in the liposome interior and the presence of PEG would prevent rapid removal of the resulting coated cationic liposomes (CCL) from circulation.

3.3 MATERIALS AND METHODS

3.3.1 Preparation of oligodeoxynucleotides

An 18-mer phosphorothioate asODN complementary to the *MDR1* gene (5'-

GTCCCCTTCAAGATCCAT-3') was synthesized by the University Core DNA Services Lab at the University of Calgary (Calgary, AB). As a sequence control, a sense phosphorothioate was also synthesized and had the sequence (5'-ATGGATCTTGAAGGGGAC-3'). In order to follow the distribution of asODN *in vivo*, the asODN was labelled with ^{125}I using Iodogen (Pierce, Rockford, IL), similar to the method described by Piatyszek *et al.*(135). ^{125}I (185 Mbq) was purchased from Amersham, Oakville, ON. The specific activity of the ^{125}I-asODN was approximately 700 kilobecquerels/μg with less than 5% free ^{125}I as determined by thin layer chromatography on PEI-cellulose (J.T. Baker, Inc., Phillipsburg, NJ). The ^{125}I label is covalently attached at the C-5 position of cytidine bases (135, 136).

3.3.2 Passively entrapped liposomal asODNs (PELA)

Neutral liposomes passively entrapping asODN (PELA) were prepared by simply hydrating a lipid film in a concentrated solution of asODN. Partially hydrogenated soy phosphatidylcholine (PC40), cholesterol (Chol) (Avanti Polar Lipids, Alabaster, AL) and polyethylene glycol$_{2000}$ distearoylphosphatidylethanolamine (PEG-DSPE) were mixed in chloroform at a molar ratio of (2:1:0.1). PC40 and PEG-DSPE were provided by SEQUUS Pharmaceuticals, Menlo Park, CA. The mixture was then dried to a thin film by rotary evaporation and traces of chloroform were removed under vacuum overnight. A solution of 1.7 mM asODN in Hepes buffer (25 mM Hepes, 140 mM NaCl, pH 7.4) was added to the lipid film to give a phospholipid concentration of approximately 300 mM and the film was allowed to hydrate for six hours at room temperature. An equal volume of asODN solution was then added and the tube was vortexed for approximately one minute. The mixture was diluted to 75 mM phospholipid with Hepes buffer and sonicated for 2 minutes. This protocol is similar to the minimal volume entrapment method described by Thierry and Dritschilo (114). The liposomes were then extruded under N_2 pressure through 200 nm Nuclepore polycarbonate filters in Hepes buffer according to Olson *et al.* (160). For experiments in which an aqueous space marker, with neutral charge, was used to follow liposomal distribution, ^{125}I-tyraminylinulin (^{125}I-TI) was prepared by the method described by Sommerman *et al.* (161), and passively entrapped using a protocol similar that described above. Liposomes were sized by dynamic light scattering using a Brookhaven BI90 particle sizer (Brookhaven Instrument, Holtsville, NY). Free ^{125}I-asODN or ^{125}I-tyraminylinulin were separated from liposomal ^{125}I-asODN/ ^{125}I-tyraminylinulin by filtration down a Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) column equilibrated with Hepes buffer.

3.3.3 Preparation of coated cationic liposomes (CCL) containing asODNs

Antisense ODNs were complexed with cationic lipids and coated with neutral lipid in the following manner: 700 µg (approximately 0.118 µmoles) of 18-mer asODN was diluted to 250 µL in distilled-deionized water (ddH$_2$O) including a trace of ^{125}I-labelled asODN. In a separate tube, 2 μmoles of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were diluted to 250 μL in $CHCl_3$ and 510 μL of CH_3OH was added. The asODN in ddH_2O was then added to the $CHCl_3/CH_3OH$ mixture containing DOTAP and the sample was mixed to form a Bligh-Dyer monophase (137). In some experiments, less asODN was added to two μmoles of DOTAP in order to alter the +/- charge ratio. Following a 30 minute incubation at room temperature, 250 μL of $CHCl_3$ was added followed by 250 μL of ddH_2O. The tube was vortexed briefly and then centrifuged at 900 x g for 7 minutes. The system then existed as a biphase and the upper, aqueous phase, was removed and the amount of asODN was determined either by radioactive counts or by measuring the absorbance at 260 nm. This procedure resulted in ~95% of the asODN being extracted into the organic phase when a 1:1 (+/-) charge ratio (DOTAP:asODN) was used. The use of a Bligh-Dyer monophase to extract plasmid DNA into an organic phase using cationic lipid has been reported previously (110, 111). PC40, Chol and PEG-DSPE, all in $CHCl_3$, were added to the organic phase to give a molar ratio of PC40:Chol:DOTAP:PEG-DSPE of 3:2:1:0.2. ddH_2O was added to give a phospholipid concentration of 20-30 mM (in ddH_2O) and the emulsion was vortexed and then sonicated for approximately one minute to form an emulsion. The organic phase was then evaporated by rotory evaporation at approximately 500 mm of Hg. The system formed a gel phase after evaporation of most of the organic phase and following further evaporation (sometimes with slight agitation), the system reverted into the aqueous phase which was briefly vortexed and residual $CHCl_3$ was evaporated.

The vesicles formed by this procedure had diameters in the range of 600 to 800 nm. They were subsequently extruded through 400 and then 200 nm polycarbonate filters. Liposomes were sized by dynamic light scattering as described above. Free asODN (if any) was separated from lipid-associated asODN by filtration down a Sepharose CL-4B column equilibrated with Hepes buffer (25 mM Hepes, 140 mM NaCl, pH 7.4).

In order to produce antibody targeted CCLs, the coupling lipid 4-(p-maleimidophenyl) butyrate - PEG-DSPE (MPB-PEG-DSPE) (custom synthesized by Shearwater Polymers, Huntsville, AL) was included at 0.5 mole% total lipid. The final lipid composition was PC40:DOTAP:PEG-DSPE:MPB-PEG-DSPE at 2:1:0.06:0.015 molar. Following extrusion in ddH_20, samples were dialyzed against at least 100 volumes of Hepes buffer for one hour. Anti-CD19 mouse mAb from mouse ascites, produced by the FMC-63 hybridoma cell line (provided by Dr. H. Zola, Children's Health Research Institute, Adelaide, Australia (162)), was purified on a Protein G column (HiTrap® Protein G, Pharmacia Biotech). A solution of 10 mg/ml antibody in Hepes buffer (25 mM Hepes, 140 mM NaCl, pH 8.0) was thiolated using Traut's reagent (2-iminothiolane) (Sigma, Oakville, ON) at a 15:1 molar ratio of Traut's:antibody for one hour at room temperature. Following incubation, free Traut's was removed by separation on a Sephadex G-50 spin column (Pharmacia Biotech) and thiolated antibody was added to CCLs at 1000:1 molar ratio lipid:antibody. The coupling reaction was carried out overnight at room temperature and the next day non-coupled antibody was separated from CCLs by passage down a Sepharose CL-4B column.

3.3.4 Animal experiments

Female ICR outbred mice (6-8 weeks old) were purchased from Charles River and used within 5 weeks of delivery at which time the weight ranged from 24 to 30 grams. Mice were given a single bolus injection via the tail vein of free or liposomal asODN in a total volume of 0.2 mL. Radioactive counts (^{125}I-tyraminylinulin or ^{125}I-asODN) for each injection ranged from 0.4 to 2 x 10^5 cpm. At specific time points (0.25, 0.5, 1, 4, 12, 24 hours) mice (3 per group) were sacrificed, and organs were dissected, weighed, and radioactive counts determined in a Beckman gamma 8000 counter. Liver, spleen, lung, heart, kidney, thyroid, and 100 µL of blood were dissected and counted, and the remainder of the animal (carcass) was also counted. The radioactive counts in each tissue and the carcass were corrected using blood correction factors which were previously determined (163). In experiments in which ^{125}I-tyraminylinulin was used as an aqueous space liposomal marker, data are presented as % of *in vivo* CPM. This corrects for leakage of the label and represents intact liposomes remaining in the body at given time points (164). Pharmacokinetic parameters were calculated using pK Analyst v1.0 (Micromath Scientific Software, Salt Lake City, UT).

3.3.5 Slot-blot method for determining pharmacokinetics

In order to confirm the pharmacokinetics of the CCL formulation, a non-radioactive method was used to measure intact asODN in the blood of mice. This method is similar to the one described by Temsamani *et al.* (165). CCL containing *MDR1* asODN without a radio-tracer were injected into the tail vein of mice which were then sacrificed at specific time points (3 mice/time point). Blood samples (100 μl) were collected and added to 400 μl of 0.5 M NaOH, 12.5 mM EDTA and then frozen. At a later time, samples were thawed, diluted further with 0.4 M NaOH/10 mM EDTA, boiled for 10 minutes and then blotted onto a Zeta-Probe® GT membrane (BioRad, Mississauga, ON) using a Bio-Dot SF slot blot apparatus (BioRad), following the protocol outlined by the manufacturer. After blotting and rinsing, the membrane was dried at 80°C for 2 hours and then stored at room temperature. Membranes were probed using a gel purified 18-mer phosphodiester ^{32}P-ODN which was complementary to the *MDR1* asODN (sODN). The probe was prepared using [γ-^{32}P]ATP (3000 Ci/mmol, DNA Core Services, University of Alberta) and T4 polynucleotide kinase (Gibco BRL, Burlington, ON), following the protocol outlined by the manufacturer. Free [γ-^{32}P]ATP was removed by separation on a BioSpin P-6 spin column (BioRad). The hybridization was carried out using ExpressHyb (Clontech, Palo Alto, CA) following the protocol outlined by the manufacturer, developed overnight, and then analysed the next day using the BioRad Model GS-250 Molecular Imager™ System (BioRad).

3.4 RESULTS

3.4.1 Effect of PEG on blood levels of cationic liposomes

It has been established that liposomes containing PEG-modified lipids (PEG-DSPE) demonstrate enhanced circulation time in blood compared to identical formulations lacking PEG (66, 69, 134). The purpose of the first set of experiments was to determine if PEG grafted to the liposome surface would increase the circulation time of cationic liposomes without asODN. Figure 3.1 compares the blood levels of liposomes containing different amounts of cationic lipid with or without PEG-DSPE at 24 hours following injection. Data in Figure 3.1 is presented as % of *in vivo* counts per minute (CPM) in blood, rather than % of injected CPM in blood. When using a non-metabolizable, aqueous-space marker such as ^{125}I-TI, leakage from liposomes results in rapid elimination of ^{125}I-TI by the kidneys. Presenting data in this way accounts for leakage and represents intact liposomes circulating in the bloodstream, allowing for a more accurate determination of the pharmacokinetics of intact liposomes (166, 167). In the absence of PEG-DSPE, all liposomes were rapidly cleared from circulation resulting in less than 5% of liposomes present in blood. For liposomes containing 50 mol% cationic lipid (DOTAP:PC40 = 1:1), inclusion of PEG-DSPE did not increase blood levels of liposomes. However, when the cationic lipid was reduced to 20 mol%, inclusion of 5 mol% PEG-DSPE significantly increased the blood levels of liposomes. At 10 mol% cationic lipid and below, PEG-DSPE had its maximum protective effect on circulation

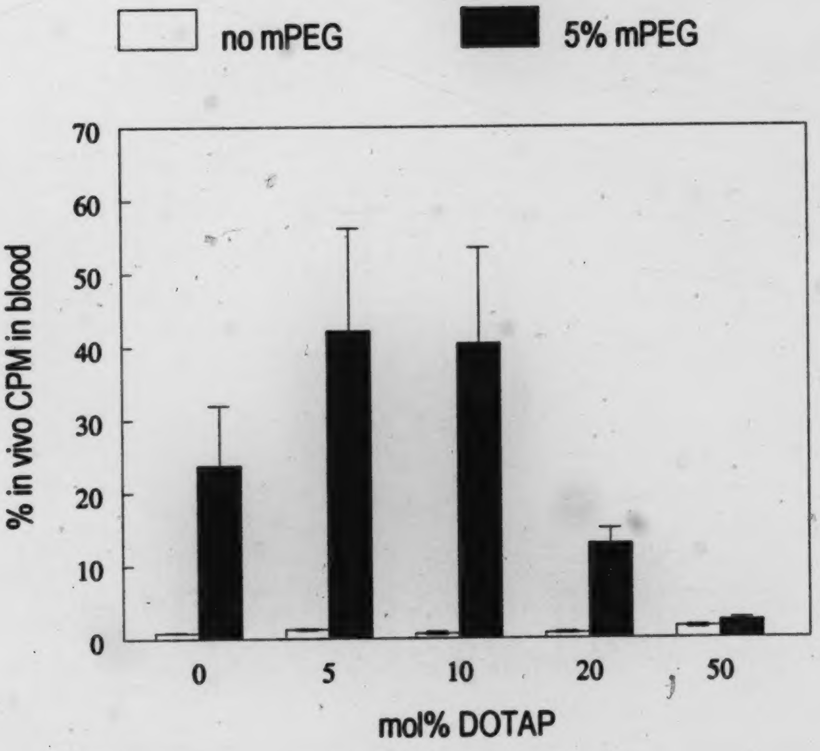


Figure 3.1: Blood levels at 24 hours post-injection of PC40 liposomes containing different percentages of DOTAP with or without mPEG. The liposomes used to generate these data were prepared by hydrating lipid films composed of PC40 and DOTAP with or without 5 mol% PEG-DSPE in a solution of ^{125}I-tyraminylinulin in Hepes buffer (25 mM Hepes, 140 mM NaCl, pH 7.4) and then extruding through 200 nm polycarbonate filters. Mice were injected via tail vein at a dose of 0.5 µmoles phospholipid/mouse in a total volume of 0.2 mL.

times, and there was no significant difference between blood levels of PEG-containing liposomes at 0, 5, and 10 mol% cationic lipid (the experiments using 5, 10, 20, and 50 mol% DOTAP were performed by Grace Kao, T.M. Allen lab). These results indicate that PEG-DSPE can increase the circulation times of liposomes containing DOTAP, particularly when 20 mol% or less DOTAP is used. Therefore, 5 mol% mPEG was added to the liposomal formulations in the subsequent experiments which examined the pharmacokinetics of cationic liposomal asODN.

3.4.2 Characterization of liposomal asODN

Following extrusion through 200 nm polycarbonate filters, the neutral liposomes had an average diameter of 197 ± 2 nm (polydispersity 0.032 ± 0.012) while the CCLs had an average diameter of 188 ± 1 nm (polydispersity 0.138 ± 0.018). The size was stable in buffer at 4°C as well as in 50% human plasma at 37°C for at least two days (Chapter 2, Figure 2.4). Entrapment of asODN within neutral liposomes resulted in approximately 20% of the added asODN associated with the lipid fraction following separation down a CL-4B column (Figure 3.2). In contrast, all of the asODN remained associated with the lipid fraction when asODNs were prepared by the CCL method (Figure 3.2).

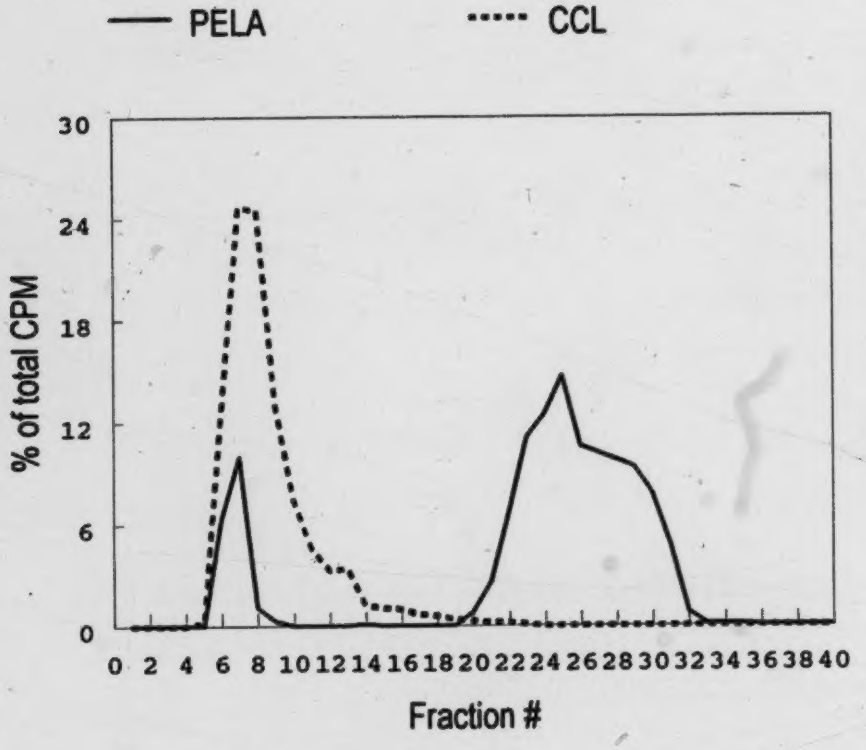


Figure 3.2: CL-4B fractionation of liposomal asODN. The first peak includes the void volume of the column and includes liposomal fraction. The second peak includes the trap volume of the column and includes free asODN.

3.4.3 Pharmacokinetics of liposomal ODN

Figure 3.3 shows the clearance from blood of both free and liposomal ^{125}I-labelled asODN injected into the tail vein of mice. Free asODN is rapidly cleared so that less than 10% of the injected dose remains in blood 30 minutes following injection. At this time approximately 40% of the injected dose has already been eliminated from the body, possibly through the kidneys (35). Most of the remaining radioactive counts exist in the carcass (undissected tissues including the bladder), liver and kidney (Figure 3.4A). At 24 hours after injection, only 8% of the initially injected dose remained *in vivo*.

^{125}I-labelled asODN encapsulated within PC40:Chol:PEG-DSPE (PELA) liposomes demonstrate a much different blood profile. PELA had an initial phase of rapid clearance which accounted for only 10% of the injected dose (Figure 3.3). The remainder was cleared with a $T_{1/2}$ of approximately 12 hours, and 24 hours following injection, almost 20% of the injected dose remained in blood. The asODN distributed primarily into the liver and spleen at all time points, and carcass levels appeared to increase at later time points (Figure 3.4B). At 24 hours following injection, approximately 45% of the injected dose remained *in vivo*.

^{125}I-labelled asODN entrapped within CCL displayed a similar profile to PELA; however, the initial clearance phase accounted for 30-40% of the injected dose (Figure 3.3). This is a result of increased uptake into the liver and spleen of a portion of the CCL dose (Figure 3.4C). This may be a consequence of some exposure of cationic lipids at the

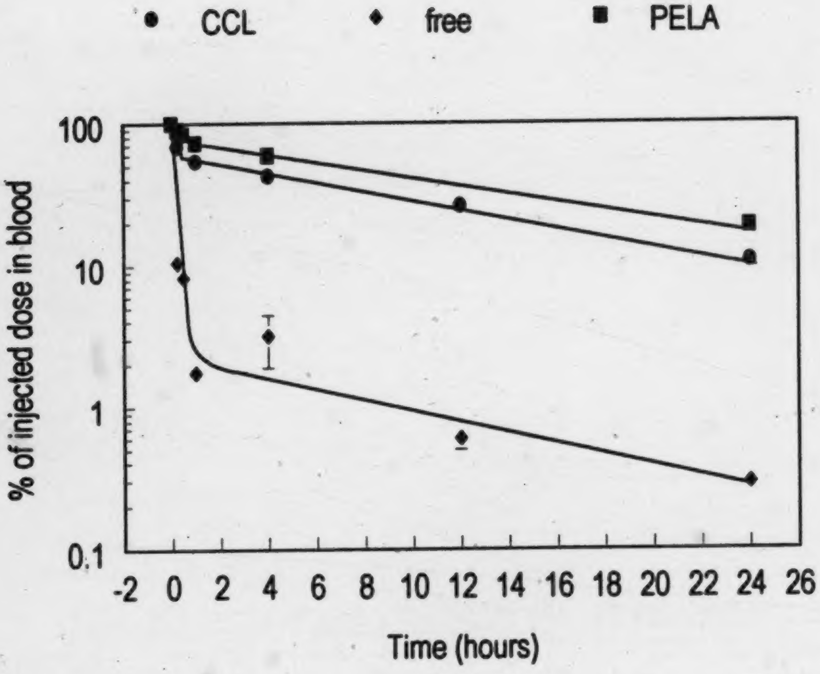


Figure 3.3: Blood levels of ^{125}I-asODN following i.v. injection into mice. Antisense ODN plus trace ^{125}I-asODN was injected in Hepes buffer (25 mM Hepes, 140 mM NaCl, pH 7.4) (●), PELA composed of PC40:Chol:PEG-DSPE, 2:1:0.1 with 6.5 µg asODN passively entrapped (◆), or 52.4 µg asODN formulated into CCL composed of PC40:Chol:DOTAP:PEG-DSPE, 3:2:1:0.2 (■). Each point represents the average of three mice ± standard deviation. In some cases the error bar is obscured by the symbol. Animals received 0.5 µmoles phospholipid/mouse in a total volume of 0.2 mL.

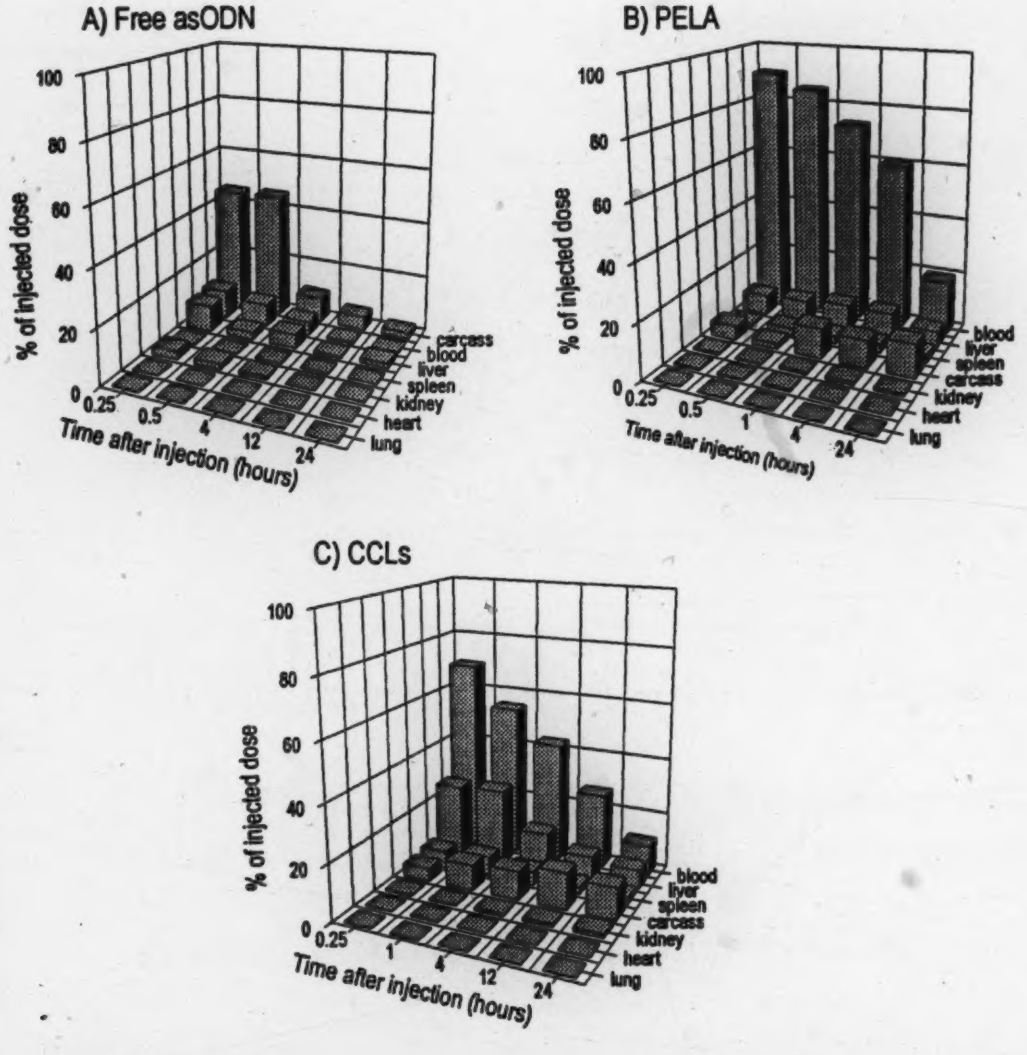


Figure 3.4: Biodistribution of asODN in mice of 3 different formulations following tail vein injection. Liposome compositions and asODN doses were the same as in Figure 3.2 and contained trace amounts of ^{125}I-asODN. A) asODN injected in Hepes buffer. B) PELA. C) CCL. Please note that the order of presentation of the tissue results changes from Figure 3.3A to 3.3B and 3C for ease of observation.

liposomal surface resulting in opsonization, with subsequent removal by cells of the mononuclear phagocyte system (MPS). The remaining dose had clearance kinetics similar to the asODN entrapped within PELA and 24 hours following injection, over 10% of the injected dose remained in the blood, and 37% remained *in vivo*. As with PELA, there was an increase in carcass levels over time, which could have resulted from either the distribution of free asODN to carcass and/or the accumulation of liposomal asODN in carcass.

Table 3.1 gives the pharmacokinetic parameters for each treatment in my experiments. Encapsulation within liposomes resulted in increased mean residence times, decreased clearance rates, as well as increased area under the time-concentration curve (AUC) for the asODN. In the first set of experiments, the injected dose was 30 μg asODN per mouse, and the results demonstrate that liposome encapsulation resulted in substantial decreases in clearance rates and increases in mean residence time and AUC, compared to free asODN. The volume of distribution (V_D) for both liposome treatments was approximately 2 mL, which is close to the blood volume of a 25 gram mouse, while the free asODN had a V_D approximately 6-fold higher, indicating that it was widely distributed within the tissues. Liposome entrapment also resulted in smaller elimination rate constants (k) and longer terminal elimination half lives ($T_{1/2}\beta$).

Table 3.1: Pharmacokinetic parameters calculated for asODN in different formulations

Group	Cl_T[a] (mL/hr)	MRT[b] (hrs)	AUC[c] (μg*hr/mL)	V_D[d] (mL)	k[e] (hr^{-1})	$T_{1/2}\alpha$[f] (hrs)	$T_{1/2}\beta$[g] (hrs)
30 μg asODN/mouse							
PELA (2.3 μmoles PL)	0.11	26.4	284.5	2.06	0.05	2.37	19.3
CCLs (0.3 μmoles PL)	0.18	17.5	166.5	2.04	0.09	0.11	12.2
Free	4.9	11.6	6.1	10.5	0.47	0.26	9.5
0.5 μmoles phospholipid/mouse							
PELA (6.5 μg asODN)	0.150	17.6	43.4	1.96	0.08	0.39	12.4
CCLs (52.4 μg asODN)	0.231	15.0	226.9	2.39	0.10	0.26	10.6

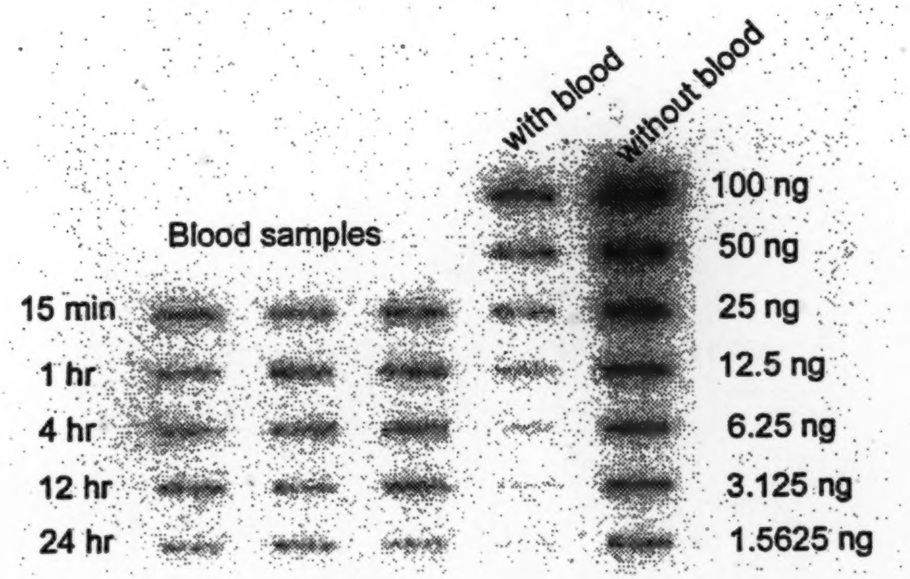
[a] Total body clearance.
[b] Mean residence time.
[c] Area under the blood concentration versus time curve.
[d] Apparent volume of distribution.
[e] Elimination rate constant from the central compartment.
[f] Half-life for initial elimination phase.
[g] Half-life for terminal elimination phase.

In the second set of experiments in Table 3.1, doses were standardized to 0.5 µmoles phospholipid per mouse. The rationale for standardizing the dose of phospholipid is that the lipid dose may have effects on liposomal pharmacokinetics and as long as the entrapped drug (^{125}I-asODN) has a slow rate of leakage, the pharmacokinetics will be governed by the liposomal carrier (167). In this case, the AUC for asODN entrapped within CCL is dramatically greater than for PELA, resulting from the increased loading efficiency. Therefore, the amount of asODN injected per µmole phospholipid is much greater, leading to a larger AUC. This is in contrast to asODN doses standardized to 30 µg asODN/mouse, in which case the AUC for PELA is greater than for asODN within CCL. The V_D and k values are similar in both cases; however, the half-lives are slightly longer for PELA at a dose of 30 µg asODN/mouse.

In preparing the asODN by the CCL procedure, I attempted to achieve charge neutrality between the cationic lipid and the anionic asODN. I hypothesized that neutralizing the DOTAP with asODN and then coating the hydrophobic particles with neutral lipid and PEG would mask the positive charge, leading to long circulation times upon injection. To test this hypothesis, I compared the MPS uptake at 15 minutes post-injection of three different CCL preparations with different amounts of added asODN for the same amount of lipid. At a 1:1 charge ratio, approximately 95% of the asODN was extracted from the aqueous phase, into the organic phase, and at this ratio, most of the positive and negative charges should be neutralized. Following the addition of neutral lipid and liposome formation via the REV procedure, all of the asODN remains associated with the lipid, and therefore I assume that the charge interaction is maintained. In this case, the asODN/PL ratio is 17.6 nmole/µmole (1.1 : 1 +/- charge ratio) and 30.4 ± 2.44% (n = 3) of the dose was taken up by the liver and spleen. However, when less asODN was associated with the CCL, so that the asODN/PL ratio was 7.5 nmole/µmole (2.6 : 1 +/- charge ratio), 41.0 ± 0.93% (n = 3) of the dose was taken up by the liver and spleen. When no asODN was added to the CCL (^{125}I-tyraminylinulin used as a marker), 49 ± 5.05% (n = 3) of the dose was taken up by the liver and spleen in 15 minutes. Therefore, I observed significantly greater uptake into the MPS tissues with increasing +/- charge ratio (One-way Analysis of Variance, $p < 0.01$) with a high degree of negative correlation between the asODN/PL ratio and the uptake into the MPS ($r^2 = 0.9998$). These results suggest that a net positive charge on the liposomes leads to greater distribution to MPS tissues.

3.4.4 Pharmacokinetics of CCL using a non-radioactive method

The literature provides several examples describing the pharmacokinetics of liposomes similar to my PELA formulation (although containing different aqueous-entrapped contents) as well as examples describing the pharmacokinetics of free phosphorothioate asODN. In order to confirm the long-circulating characteristics of the CCL formulation, a slot-blot method was used to measure intact asODN in the blood of mice injected i.v. with non-radioactive asODN within CCL. Blood levels of asODN can be quantified by running a series of standards of asODN diluted in blood and constructing a standard curve. Figure 3.5 is a phosphorimage of a membrane blotted with a series of test samples (3 per time point) as well as known amounts of asODN diluted in blood, or buffer. The presence of whole blood dramatically diminishes the signal for asODN, and therefore samples diluted in blood were analysed by densitometry and the areas under the peaks were used to construct a standard curve, Figure 3.6. Test samples were also analysed by densitometry and converted into ng asODN using the equation for the standard curve. Figure 3.7 illustrates the results for the blood pharmacokinetics determined using this method compared to the results using ^{125}I-asODN as a radio-tracer (same data as in Figure 3.3). The profiles are quite similar at all time points and these data confirm that a significant proportion of intact and functional asODN formulated in CCL remains in blood, and 24 hours after injection, 10% of the injected dose is still in circulation.



Phosphorimage of blood samples from mice injected with non-radiolabelled asODN membrane hybridized with ^{32}P-probe

with blood
without blood
Blood samples
15 min
1 hr
4 hr
12 hr
24 hr
100 ng
50 ng
25 ng
12.5 ng
6.25 ng
3.125 ng
1.5625 ng

Figure 3.5: Phosphorimage of blood samples of mice injected with asODN formulated in CCL, blotted onto a nylon membrane and then hybridized with a ^{32}P-labelled probe. Three mice per time point were sacrificed and blood samples collected, processed as described in the Methods section and then blotted onto a nylon membrane. Two sets of standards were also run by adding known amounts of asODN to blood, or buffer.

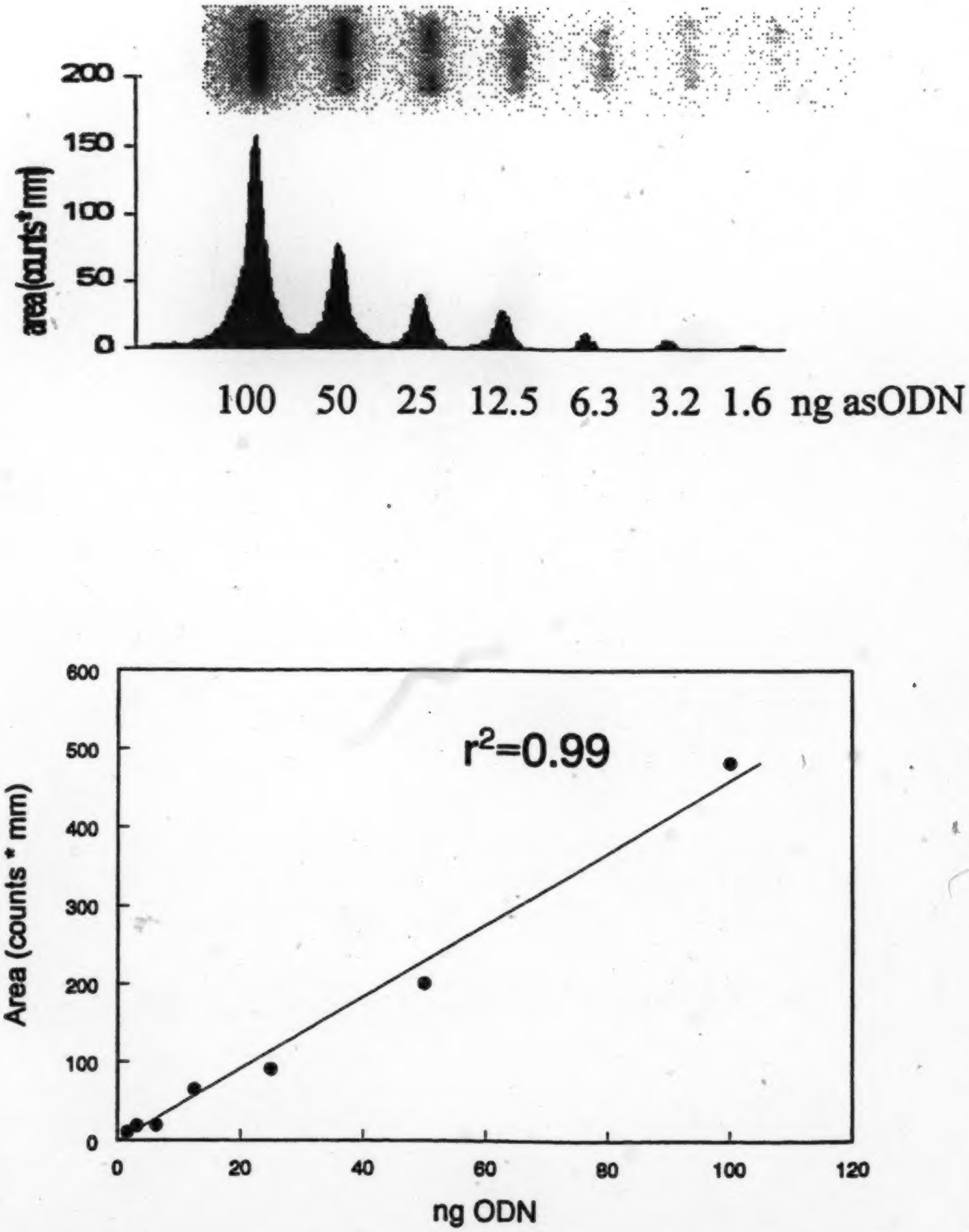


Figure 3.6: Densitometric analysis and standard curve generated using the asODN standards diluted in blood. The phosphorimage of the standards was taken from ~~the~~ Figure 3.5.

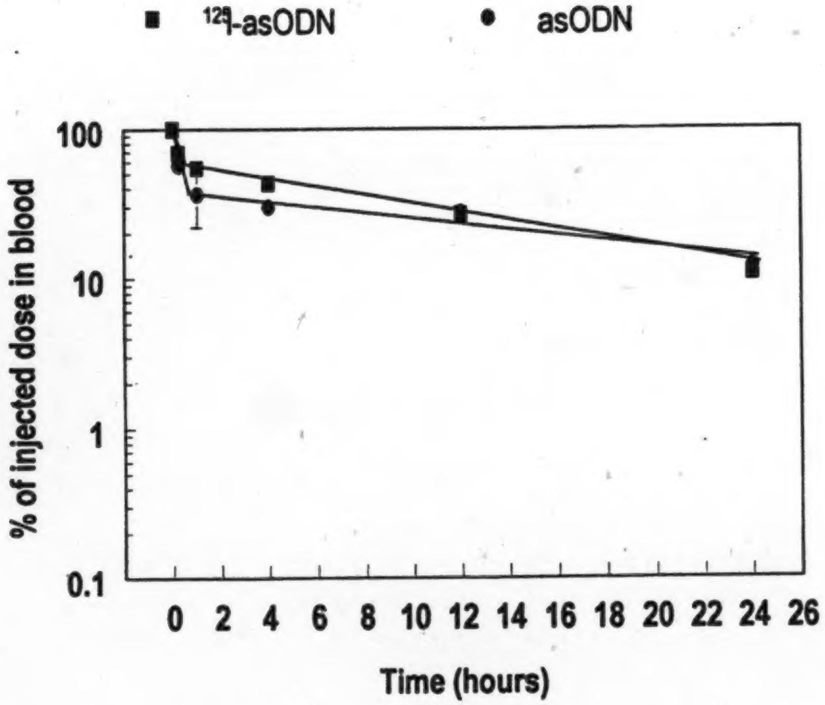


Figure 3.7: Blood levels of non-radiolabelled asODN (●), or ^{125}I-asODN (■) formulated in CCL. Values for non-radiolabelled asODN were calculated using the data in Figure 3.5, while values for ^{125}I-asODN are from Figure 3.3. Each point represents the mean ± standard deviation from three different experiments.

3.5 DISCUSSION

This study examined the pharmacokinetics of asODN formulated in a novel liposomal carrier which is designed to have a high loading efficiency, to be of small size (<200 nm), and to be able to circulate in the bloodstream for extended times. My results indicate that the formulation has long-circulating characteristics similar to asODN passively entrapped within non-cationic liposomes. I have confirmed the pharmacokinetics of asODN formulated in CCL by a non-radioactive method as well. The slot-blot hybridization method confirmed that the asODN formulated within CCL represents intact and functional asODN.

AsODN have poor pharmacokinetics which could prevent therapeutic concentrations from being reached in target tissues. When injected intravenously, free asODNs leave the circulation rapidly, extravasate into tissues, and then a great proportion is excreted in the urine. Studies examining the biodistribution of phosphorothioate ODNs demonstrate a wide variation in the actual pharmacokinetic values; however they all indicate that asODNs have a biphasic pattern of elimination from blood, consistent with my current results (35, 38). The initial distribution phase has been reported to have a $T_{1/2}$ ranging from less than one minute to over 30 minutes, and then a longer elimination phase ranging from less than one hour to over 40 hours. These studies and others demonstrate that the kidney and liver are the major organs of uptake, and that these are the major sites of metabolism of asODNs (14, 35, 38). In a recent review, Tonkinson and Stein suggest that the concentrations of phosphorothioate ODNs reached in the serum of human patients during an infusion (0.32-0.97 µM) during a phase I clinical trial, would not be high enough to begin to detect clinical responses (14, 168).

Antisense ODNs could benefit from the use of a long-circulating liposomal carrier *in vivo* for several reasons. Entrapment within liposomes could increase the distribution of asODN to target tissues and away from sites of metabolism and excretion. Phosphorothioate asODNs are modified to resist degradation by nucleases through the replacement of a non-bridging oxygen atom by a sulphur atom in the phosphodiester bond. However, nuclease resistance is not absolute, and studies have shown that significant metabolism occurs (35, 120). Liposomes could provide a means of nuclease protection in the circulation as well as in tissues. Antisense-ODNs carry a strong negative charge, and therefore do not readily interact with, or cross, cell membranes. Association with cationic lipids is one way to overcome this problem; however, the cellular association and uptake mediated by cationic lipids is not cell-specific.

The present study indicates that cationic lipids provide a means of achieving efficient loading of asODN into liposomes and are therefore a desirable component of liposomal asODN formulations. However, the positive charge should be neutralized by asODN or shielded by coating lipid to prevent interaction with non-target cells or opsonization by serum proteins which leads to removal by cells of the MPS. I have used a procedure in which cationic lipid-asODN particles are coated with neutral lipids and a

PEG-modified lipid which will add stability and increase circulation time upon administration. In addition, coupling lipids can be added to the coating lipid and, following liposome formation, cell-specific targeting ligands can be attached onto the surface to produce a targeted carrier.

I have used cholesterol and partially hydrogenated phosphatidylcholine (PC40) as helper lipids in the formulations, rather than DOPE which has recently been shown to be unnecessary for *in vivo* transfection using plasmid DNA (106, 107). Those studies demonstrated that cholesterol was a much better helper lipid than DOPE when assessing the expression of a reporter gene following injection of plasmid DNA-cationic liposome complexes (lipoplexes). Both of the liposomal asODN formulations used in my experiments contained mPEG-DSPE at 5 mol%; past studies have shown that PEG-DSPE decreases uptake into the liver and spleen, thereby increasing the circulation time of liposomes in blood (66, 69, 134). In the study by Hong *et al.* it was found that lipoplexes containing PEG-PE were active in transfecting cells *in vivo*, even following long-term storage (106).

There is a striking decrease in the rate of clearance of the ^{125}I-asODN within CCL compared with other studies following the pharmacokinetics of asODN complexed with cationic liposomes. Past studies have demonstrated that the asODN-lipoplexes leave the blood rapidly, accumulating in the liver (120), and in one study embolism was suggested to account for a transient accumulation in the lung (121). Since cationic lipids have been shown to be toxic *in vitro* as well as *in vivo*, accumulation in these tissues could lead to problems, especially after repeat administration (169, 170). In both of these studies cationic lipoplexes were used with no attempt to modify the formulation for use *in vivo*.

Litzinger *et al.* examined the biodistribution following injection into mice of an ^{125}I-labelled 18-mer phosphorothioate oligonucleotide complexed at a 4:1 (+/-) charge ratio with cationic liposomes composed of DC-Chol and DOPE (121). The lipoplexes which formed had a size greater than 1800 nm and following injection they accumulated in the lung within 5 minutes, followed by a gradual redistribution to the liver which accounted for the greatest proportion of the injected dose five hours following injection (121). The blood clearance profile was biphasic, with an initial $T_{1/2}$ of less than 5 minutes and a terminal $T_{1/2}$ of approximately 5-6 hours. This pattern of distribution resulted in less than 5% of the injected dose existing in the blood one hour following injection, and much less than 1% at 24 hours.

Bennet *et al.* examined the biodistribution of a ^{3}H-labelled phosphorothioate asODN formulated with the cationic liposomes composed of DMRIE and DOPE (1:1 molar ratio) at a charge ratio of 2.5:1 (+/-) (120). Similar to the study by Litzinger *et al.*, the liver (~70%) and lung (~20%) contained the greatest proportion of the injected asODN one hour post-injection. These studies used formulations which are optimized for delivery of asODN to cells *in vitro*, and some considerations should be made in tailoring a liposome formulation for use in delivering asODNs *in vivo*.

As described in the Methods section, an effort was made to titrate the amount of cationic lipid required to complex the asODN so that there was no excess positive charge. I hypothesized that neutralizing the positive charge with negatively charged asODN would ultimately lead to a decrease in interaction of the cationic lipid with opsonins, which could mediate uptake of liposomes into MPS tissues. My results indicate that if the balance of positive to negative charge is upset, such that there is more positive charge (DOTAP) in the formulation than negative charge (asODN), there is a significantly greater distribution to these MPS tissues. This interpretation is supported by a study which demonstrated the reversal of a net positive charge on cationic liposomes to net negative following incubation in serum, indicating extensive interaction with serum proteins (171). Other studies indicate that higher concentrations of serum are required to inhibit the cellular interaction of cationic liposomes lacking asODN, than were required to inhibit the interaction when asODN were present in the formulation (121).

Neutral liposomes, containing ^{125}I-asODN passively entrapped, avoid uptake into MPS tissues to a greater extent than cationic liposomes. However, the major draw-back to using neutral liposomes is the inefficient loading of asODN within the aqueous space. As figure 3.2 illustrates, only about 20% of the added asODN remains associated with the 200 nm liposomes following separation down a CL-4B column, and this loading could be expected to decrease with decreasing vesicle diameter. This results in loss of expensive asODN, or necessitates a time-consuming recovery step which may prevent the use of this formulation at a clinical level. In addition, the low loading efficiency makes the dose of lipid per dose of asODN much greater.

In contrast, virtually 100% of the added asODN remains associated with liposomes containing DOTAP prepared by the extraction-REV procedure. This illustrates one of the major advantages of cationic lipids in liposomal-asODN formulations, namely that the electrostatic interaction results in very efficient loading. The other advantage, which is exploited *in vitro*, is that excess positive charge will facilitate the delivery of asODN through interaction with the negatively charged cell membrane. However, if the pharmacokinetics of cationic liposomes are to be controlled *in vivo*, and tissues outside of the MPS are to be targeted, excess positive charge will be an undesirable characteristic (172). Therefore, if cationic liposomes are to be used to deliver asODNs *in vivo*, some effort must be made to optimize the formulation so that it can reach the target tissue. I have demonstrated that, when formulated in a charge-neutralized fashion, cationic liposomal-asODN complexes coated with neutral lipids can circulate in blood for extended times. This may open the opportunity for passive targeting to solid tumours as seen with Stealth® liposomal drugs such as doxorubicin (173, 174). The ability of liposomes to maintain asODNs in the blood stream presents the opportunity for ligand-mediated targeting to haematological diseases, as recently demonstrated in our laboratory (88).

CHAPTER 4

Targeting a multidrug-resistant human B-cell lymphoma using a novel liposomal formulation of *MDR1* antisense oligodeoxynucleotides

4.1 ABSTRACT

Inhibiting the expression of specific genes using antisense oligodeoxynucleotides (asODN) is an interesting and novel approach to treating disease. The specificity is encoded by the base sequence and therefore asODN can be tailored to hybridize only to the target mRNA. This, combined with the relatively non-toxic nature of these molecules, make asODN promising drug candidates. However, asODN have not yet found wide-spread use in therapeutic applications and one of the most frequently cited concerns has been the question of delivery. To date, no suitable carrier has been developed for systemic administration of asODN. I have developed a novel liposomal formulation of asODN, coated cationic liposomes (CCL), which has a high loading efficiency for asODN, is stable in plasma, and circulates in the bloodstream for extended times following i.v. administration. In this paper, I assess the ability of CCL to deliver asODN targeted to the multidrug-resistance gene, *MDR1*, which codes for P-gp and is responsible for the multidrug resistant phenotype characteristic of cancer cells. In addition, I was able to attach a mAb onto the surface CCL and evaluate the effectiveness of a targeted carrier. My results indicate that targeting results in significant increases in the quantity of asODN delivered to cells. Results from antisense experiments indicate that asODN delivered using targeted CCL causes decreases in P-gp activity; however, this could not be corroborated by decreases in *MDR1* mRNA levels or by increased sensitivity to doxorubicin. In spite of the equivocal data obtained in the antisense experiments, I have

demonstrated that CCL can be targeted by attaching a mAb, and this may represent a useful delivery strategy for asODN.

4.2 INTRODUCTION

Antisense oligodeoxynucleotides (asODN) are designed to be highly specific therapeutic agents. The base sequence of the asODN is complementary to a sequence on the mRNA of the target gene and, if the antisense sequence is long enough, (e.g., 18 bases) then the asODN should hybridize only to that mRNA. An 18 base sequence should occur only once every 6.9×10^{10} (4^{18}) bases and, assuming that the haploid human genome contains 3×10^{9} bases, an 18-mer asODN should be complementary to only one mRNA (175). This specificity, combined with the relative lack of toxicity compared to other drugs, make asODN very attractive drug candidates. However, there are several limitations to the practical use and ultimate success of asODN in the treatment of human diseases. AsODN must reach the target cell population and the correct intracellular compartment to hybridize effectively a significant proportion of the target mRNA. The cytoplasmic membrane is a significant barrier, since asODN cannot passively diffuse through it. Studies have shown that asODN can enter cells via adsorptive or fluid-phase endocytosis (176-178) and cell-surface receptors which bind asODN have been identified; however, their capacity to transport actively sufficient asODN intracellularly, in different cell types, is not clear (28, 179).

In vitro, cationic liposomes have been shown to increase dramatically the intracellular delivery and activity of asODN and in some cases are an absolute requirement for antisense activity (94). *In vivo* the requirement for a lipid carrier is much less apparent since there are several examples in animal models and human clinical trials which demonstrate the therapeutic activity of free asODN (22, 23, 150). It is not clear why free asODN would be active *in vivo* but often require a carrier *in vitro*. For example, phosphorothioate asODN had to be complexed with Lipofectin to inhibit the expression of intracellular adhesion molecule-1 (ICAM-1) *in vitro*, while free asODN administered by i.v. infusion demonstrated significant activity in human clinical trials involving antisense inhibition of ICAM-1 (23).

It is difficult to draw conclusions regarding the usefulness of liposomes for delivering asODN *in vivo*. There are published examples of *in vivo* use of liposomes to deliver asODN; however, most of these involve local administration to tissues such as the myocardium (115), the carotid artery (24), and ligaments of damaged joints (125). There are very few examples of i.v. administration of liposomal asODN and for many diseases, this will be the most convenient route of administration. Gokhale *et al.* used an asODN directed against *raf-1* and found that i.v. administration of the asODN associated with cationic liposomes resulted in decreases in Raf-1 protein in the liver, kidney and tumour xenografts of mice (123). However, i.v. administration of free asODN resulted in no decreases in Raf-1 protein in any of the tissues examined. This example demonstrates the potential usefulness of a liposomal carrier to deliver asODN to tissues *in vivo* following i.v. administration.

The formulation used by Gokhale *et al.* did not consist of typical cationic lipoplexes, which are formed by mixing pre-formed cationic liposomes with asODN. Rather than using DOPE, which is frequently used as a helper lipid, they combined the cationic lipid with phosphatidylcholine and cholesterol and hydrated the dried lipid mixture in a solution containing the asODN. This formulation was shown to result in increased blood levels compared to free asODN following i.v. administration in mice; however, the circulation time was still quite short and there was extensive uptake by the liver.

The study by Gokhale *et al.* is significant since it demonstrates that alternative cationic liposome formulations can be active *in vivo*; however, the liver uptake and short circulation times in blood could be a limitation to their use. In an effort to improve on their formulation, I have developed a formulation called coated cationic liposomes (CCL). CCL are made with just enough cationic lipid to load efficiently the asODN with emphasis on trying to balance the positive and negative charges. I have demonstrated that CCL efficiently load asODN, can be extruded to small diameters (< 200 nm), are stable in human plasma, and have a favourable pharmacokinetic profile compared to cationic lipoplexes.

In order to evaluate the activity of the CCL formulation I have used a phosphorothioate *MDR1* asODN designed to inhibit the expression of the *MDR1* gene in a multidrug-resistant human B-lymphoma cell line (Namalwa/MDR$_{40}$). Multidrug-resistance (MDR) is a phenomenon which sometimes occurs in malignant cells and results in cross-resistance to a range of chemotherapeutic agents. MDR often results from the overexpression of the *MDR1* gene which codes for P-gp (180). P-gp is a 170 kDa glycoprotein which consists of two homolgous halves, each half with a large intracytoplasmic loop and six transmembrane domains (reviewed in (181)). The presence of P-gp on the surface cancer cells causes resistance to a range of chemotherapeutic agents as a result of its ability to pump anticancer drugs out of cells in an ATP-dependent manner. The existence of P-gp in malignant tissue results in reduced efficacy in treating the malignancy and ultimately translates into a less favourable patient prognosis (181).

Overexpression of *MDR1* may occur via gene amplification (182) and factors involved in the regulation of *MDR1* at the transcriptional level have been identified and may contribute to the MDR phenotype (183, 184). For example, anticancer agents such as vincristine and adriamycin have been shown to activate directly the promoter of the *MDR1* gene (185) and other studies have demonstrated that increased expression of *MDR1* can occur without gene amplification (186). Several strategies have been used in an attempt to decrease P-gp mediated MDR, including inhibitors such as verapamil and cyclosporin A derivatives (187-189) as well as strategies aimed at inhibiting the translation of *MDR1* mRNA using ribozymes or asODN (reviewed in (190)).

One of the most notable examples of *MDR1* modulation by asODN was described by Cucco and Calabretta (150). They demonstrated inhibition of *MDR1* by an 18-mer phosphorothioate asODN which overlaps the initiation codon and includes codons 1-6 of the *MDR1* gene. *In vitro*, the asODN decreased the amount of *MDR1* mRNA in MDR HL-60 cells and increased their sensitivity to vincristine. When these cells were injected i.v. into SCID mice, followed by i.v. administration of asODN (or scrambled control), and i.v. treatment with vincristine, asODN treatment resulted in 6/10 long-term survivors, while there were no long-term survivors in the other groups (scrambled ODN or no ODN with or without vincristine).

There are several published examples in which asODN have been used to inhibit the expression of *MDR1* (132, 150, 191-196) and therefore this appeared to be an appropriate system in which to evaluate asODN delivery using the CCL formulation. The Namalwa/MDR$_{40}$ cells which overexpress *MDR1* also provide a good model in which to study the effectiveness of targeted-CCL for delivering asODN. These cells express CD19 on their surface which internalizes upon binding to αCD19 mAb and this has proved to be a useful system to study the intracellular delivery of doxorubicin to the parental cell line (Namalwa) using αCD19-targeted liposomes (88, 197). This chapter describes the characterization of the Namalwa/MDR$_{40}$ cell line, the ability of different liposomal formulations to deliver *MDR1* asODN to these cells, and modulate the expression of this gene.

4.3 MATERIALS AND METHODS:

4.3.1 Materials

Partially hydrogenated egg phosphatidylcholine (PC40) and polyethylene glycol$_{2000}$ distearoylphosphatidylethanolamine (PEG-DSPE) were provided by SEQUUS Pharmaceuticals, Inc. (Menlo Park, CA). Cholesterol (Chol) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL). 4-(p-maleimidophenyl) butyrate - PEG-DSPE (MPB-PEG-DSPE) was custom synthesized by Shearwater Polymers (Huntsville, AL). LipofectAMINE®, RPMI 1640, fetal bovine serum (FBS), agarose (Ultra Pure), 3-[N-morpholino] propanesulfonic acid (MOPS) buffer, T4 polynucleotide kinase, *Eco*RI and TRIzol™ were all purchased from Gibco BRL (Burlington, ON). Proteinase K, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and 2-iminothiolane (Traut's reagent) were purchased from Sigma (Oakville, ON). Doxorubicin was obtained from Adria Laboratories Inc. (Mississauga, ON). Calcein-acetoxymethyl ester (calcein-AM) was purchased as part of the Vybrant™ Multidrug-Resistance Assay Kit from Molecular Probes (Eugene, OR). ExpressHyb™ hybridization solution was purchased from Clontech (Palo Alto, CA). Methanol, chloroform and sodium chloride were from BDH Inc. HEPES was purchased from Fisher Scientific (Nepean, ON).

4.3.2 Cell Lines and antibodies

The human B-cell lymphoma line, Namalwa (ATCC CRL 1432) was obtained from American Type Culture Collection (Rockville, MD) and grown as suspension cultures in RPMI 1640 supplemented with 10% FBS at 37°C in a humidified incubator (90% humidity) with 5% CO_2. The Namalwa/MDR_{40} cell line was derived from the Namalwa line through transfection with a plasmid containing the *MDR1* gene, pHaMDR1a (a generous gift from M. Gottesman, National Institutes of Health, Bethesda, MD) using LipofectAMINE®. Transfected cells were then selected by adding 2 ng/mL colchicine to the media three days following transfection. Six days following transfection, the concentration was increased to 3 ng/mL and then to 4 ng/mL 12 days after transfection. A sample of cells were split off at this point and grown in 4 ng/mL vincristine. Twenty-five days following transfection, the vincristine and colchicine concentration was increased to 8 ng/mL and at this concentration, control cells, which were not transfected, did not proliferate. By 70 days following transfection, the vincristine/colchicine concentration was increased to 40 ng/mL and at this point a sample of cells were split off and maintained at this concentration of vincristine (Namalwa/MDR_{40} cells). The Namalwa/MDR_{100} cell line was selected by continuing to increase the colchicine/vincristine concentration up to 100 ng/mL (transfection and selection for both lines was carried out by Susan Cubitt, Allen Lab, University of Alberta). The CEM/VLB_{100} cell line (a generous gift from W. Beck, University of Illinois at Chicago) is a human T-cell lymphoblastic leukemia cell line which was derived from the parent line (CCRF-CEM) by selection in increasing concentrations of vinblastine (198) and was grown under the same conditions as the Namalwa cells in 100 ng/mL vincristine.

Anti-CD19 mAb was purified from mouse ascites produced by the intraperitoneal injection of the hybridoma cell line, FMC-63 (a generous gift from Dr. H. Zola, Children's Health Research Institute, Adelaide, Australia (162)). Ascites fluid was collected by the staff of the animal care facility in the Heritage Medical Research Centre and, following the removal of blood cells (centrifugation) and lipid (Sep-Pac Cartridges, Waters, Mississauga, ON), antibody was purified (by Susan Cubitt and Rene LeClare, Allen Lab, University of Alberta) on a HiTrap® Protein G column (Amersham Pharmacia Biotech, Baie d'Urfe, PQ). The anti-P-gp mAb (MRK16) was kindly provided by Takashi Tsuro (Cancer Chemotherapy Center, Tokyo, Japan) (199). Goat anti-mouse FITC secondary antibody for flow cytometry was purchased from Sigma (Oakville, ON).

4.3.3 Oligodeoxynucleotides

An 18-mer phosphorothioate asODN complementary to the *MDR1* gene (5'-GTCCCCTTCAAGATCCAT-3') was synthesized by the University Core DNA Services Lab at the University of Calgary (Calgary, AB) and was the same sequence as used by Cucco and Calabretta (150). As a sequence control, a sense (sODN) phosphorothioate was also synthesized with the sequence (5'-ATGGATCTTGAAGGGGAC-3'). A gel-purified 18-mer phosphodiester sODN was purchased from the same source and used as a probe for hybridization in the slot blot experiments described above.

4.3.4 Preparation of liposomes

Antisense ODNs were complexed with cationic lipids and coated with neutral lipid in the following manner: 700 µg (approximately 0.118 µmoles) of 18-mer asODN/sODN was diluted to 250 µL in distilled-deionized water (ddH_2O). In a separate tube, 2 µmoles of DOTAP were diluted to 250 µL in $CHCl_3$ and 510 µL of OH was added. The asODN in ddH_2O was then added to the $CHCl_3/CH_3OH$ mixture containing DOTAP and the sample was mixed to form a Bligh-Dyer monophase (137). Following 30 minutes incubation at room temperature, 250 µL of $CHCl_3$ was added followed by 250 µL of ddH_2O. The tube was vortexed briefly and centrifuged at 900 x g for 7 minutes. The system then existed as a biphase and the upper, aqueous phase, was removed and the amount of asODN was determined either by radioactive counts or by measuring the absorbance at 260 nm. This procedure resulted in ~95% of the asODN being extracted into the organic phase when a 1:1 (+/-) charge ratio (DOTAP:asODN) was used. The use of a Bligh-Dyer monophase to extract plasmid DNA into an organic phase using cationic lipid has previously been reported (110, 111). PC40 and PEG-DSPE in $CHCl_3$, were added to the organic phase to give a molar ratio of PC40:Chol:DOTAP:PEG-DSPE of 3:2:1:0.2. ddH_2O was added to give a phospholipid concentration of 20-30 mM (in ddH_2O) and the emulsion was vortexed and then sonicated for approximately one minute to form an emulsion. The organic phase was then evaporated by rotary evaporation at approximately 500 mm of Hg. The system formed a gel phase after evaporation of most of the organic phase, and following further evaporation (sometimes with gentle mixing), the system reverted into the aqueous phase which was briefly vortexed and residual $CHCl_3$ was evaporated.

The vesicles formed by this procedure had diameters in the range of 600 to 800 nm. They were subsequently extruded through 400 and then 200 nm polycarbonate filters (Nuclepore Corp., Pleasanton, CA). Liposomes were sized by dynamic light scattering using a Brookhaven BI90 particle sizer (Brookhaven Instruments, Holtsville, NY).

In order to produce antibody targeted CCLs, the coupling lipid MPB-PEG-DSPE was included at 0.5 mol% of total lipid. The final lipid composition was PC40:DOTAP:PEG-DSPE:MPB-PEG-DSPE at a 2:1:0.06:0.015 molar ratio. Following extrusion in ddH_20, samples were dialyzed against at least 100 volumes of HEPES buffer (25 mM HEPES, 140 mM NaCl, pH 7.4) for one hour. A solution of 10 mg/mL αCD19 antibody in HEPES buffer (pH 8.0) was thiolated using Traut's reagent at a 15:1 molar ratio of Traut's:antibody for one hour at room temperature. Following incubation, free Traut's was removed by separation on a Sephadex G-50 spin column (Pharmacia Biotech, Uppsala, Sweden) and thiolated antibody was added to CCLs at 1000:1 molar ratio of lipid:antibody. The coupling reaction was carried out overnight at room temperature and the next day non-coupled antibody was separated from CCLs by passage down a

Sepharose CL-4B column.

4.3.5 Treatment of cells

Cells were seeded at 0.5 x 10^6 cells/mL in medium containing 10% FBS and approximately 1 μM asODN or sODN delivered using αCD19-CCL, CCL, LipofectAMINE®. In some experiments a high concentration of free asODN (20 μM) was tested. At 24, 48, and 72 hours the number of cells was determined using a Coulter counter and enough fresh medium and asODN/sODN treatment was added to dilute the cells back to 0.5 x 10^6 cells/mL. Cytotoxicity assays performed earlier indicated that the concentrations of asODN/sODN and carrier were not toxic and the Coulter counter measurements confirmed that doubling times were not different than for non-treated cells.

4.3.6 Northern Analysis

Total RNA from cells was isolated using TRIzol™ following the protocol outlined by the manufacturer. Briefly, 6-10 x 10^6 cells were collected into 15 mL conical tubes, centrifuged at 300 x g for 7 minutes, and then resuspended in 1 mL TRIzol™. The mixture was transferred to a 1.5 mL microfuge tube and then 0.2 mL $CHCl_3$ was added and the tubes were mixed by hand. Phases were separated in a microcentrifuge at 12,000 x g for 15 minutes at 4°C and then the upper aqueous phase was removed and placed in another 1.5 mL microfuge tube. Isopropanol (0.5 mL) was added and after 10 minutes at room temperature, RNA was pelleted in a microcentrifuge at 12,000 x g for 10 minutes. The RNA pellet was rinsed twice with 1 mL 75% ethanol, dried at room temperature and resuspended in 50 µl diethyl pyrocarbonate-treated water. Samples were stored over short times at 4°C (1-2 weeks) with long-term storage at -80°C.

Denaturing agarose-formaldehyde gel electrophoresis was carried out using standard procedures (200). RNA concentrations were determined spectrophotometrically by absorbance at 260 nm, and 17 µg samples were aliquoted into 1.5 mL microfuge tubes. Samples were concentrated to volumes less than 10 µl and then diluted in RNA sample buffer (6% deionized formaldehyde, 50% deionized formamide, 20 mM MOPS in DEPC-water) up to a volume of approximately 30 µl. After heating to 65°C for 15 minutes and cooling on ice, 3 µl loading dye (25% glycerol, 0.35 µM EDTA, bromophenol blue and xylene cyanol in DEPC-water), and 0.5 µl ethidium bromide (10 mg/mL in DEPC-water) were added. A 1.5% agarose gel in MOPS running buffer (1 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0) containing 2.3 % formaldehyde was set in an 11 x 14 cm tray. MOPS buffer was added to the electrophoresis box, samples were loaded, and the gel was electrophoresed for 3-4 hours at approximately 90 volts.

The gel was rinsed for one hour in DEPC-water and then RNA was blotted onto a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) overnight using standard procedures. The next day, the filter was dried for 2 hours at 80°C under vacuum and then stored at room temperature. Membranes were probed for *MDR1* RNA using an *MDR1* cDNA which was removed from the plasmid, pHDR5A (American Type Culture Collection). The plasmid was digested with *Eco*RI for one hour at 37°C and electrophoresed on a 1% agarose gel. The 1.38 kb band was cut out of the gel and the DNA was recovered using a QIAEX II DNA extraction kit (QIAGEN Inc., Santa Clara, CA). ^{32}P-*MDR1* probes were prepared using [α-^{32}P]dCTP (3000 Ci/mmol, DNA Core Services, University of Alberta) and an oligolabelling kit (Amersham Pharmacia Biotech). Free [α-^{32}P]dCTP was removed using Bio-Spin 30 spin columns (BioRad, Mississauga, ON). Membranes were prehybridized for 0.5 - 1 hour in ExpressHyb™ (Clontech, Palo Alto, CA), and then hybridized for 1 - 18 hours with ^{32}P-probe. Membranes were washed according to the protocol outlined by the manufacturer and then developed overnight on a BioRad Model GS-250 Molecular Imager™ System (BioRad). Membranes were stripped for 30 minutes (0.05% SDS, 90-100°C) and then re-probed using a ^{32}P-β-actin probe which was prepared using the procedure outlined above for the *MDR1* probe.

4.3.7 Flow cytometry

Cells were analysed for CD19 and P-gp by incubating 1 x 10^6 cells in 50 μl of PBS containing 10 μg anti-CD19 mAb or anti-P-gp mAb (MRK-16), in 12 x 75 mm polystyrene tubes. Following a 30 minute incubation at 4°C, the tubes were centrifuged at 300 x g, the supernatant was aspirated and cells were resuspended in 50 μl of a 1:50 dilution of goat anti-mouse FITC secondary antibody in PBS. Following a 45 minute incubation at 4°C, cells were washed 3 times with cold PBS and then resuspended in 0.5 mL 1% formalin. Samples of 10,000 events were collected using a Becton Dickinson FACScan Flow Cytometer and data was analysed using the LYSIS II software program (Becton Dickinson, San Jose, CA).

4.3.8 Calcein accumulation assay

The calcein accumulation assay was carried out basically as described by the manufacturer (Molecular Probes, Eugene, OR). Following different treatments (asODN, sODN, etc.), cells were transferred to a 96-well plate, 5 x 10^6 cells per well, 5 wells per group. Calcein-AM was added to give a final concentration of 0.25 µM and the plate was incubated for 15 minutes at 37°C. Following incubation, cells were washed three times in cold PBS and then resuspended in 0.2 mL cold tissue culture medium. Calcein-specific fluorescence was measured in a Millepore Cytofluor 2300 plate reader with a 485 nm excitation filter and a 530 nm emission filter. Background fluorescence (wells without calcein-AM added) was subtracted from the fluorescence value for each well.

4.3.9 Cytotoxicity Experiments

Doxorubicin sensitivity was evaluated following the asODN/sODN treatments using a cell proliferation assay which measures the metabolic conversion of a tetrazolium dye (201). Cells were seeded at 1 x 10^5 per well in 96-well plates and a range of doxorubicin concentrations were added. Following a one hour incubation at 37°C, each well was washed 3 times in complete medium and then incubated for an additional 47 hours. Next, the medium was replaced with 50 µl of complete medium containing 0.5 mg/mL MTT and cells were incubated for an additional 4 hours at 37°C. One hundred microlitres of acid isopropanol (0.04 M) was added per well and the plates were mixed for 20 minutes in order to dissolve the formazan dye product. The absorbance for each well was measured at dual wavelengths (570 nm and 650 nm) on a Titertek Multiskan Plus (Flow Laboratories Inc., Mississauga, ON). Absorbance values were expressed as percent of control (no doxorubicin treatment), and the average of at least three wells was used at each doxorubicin concentration to construct the concentration-response curves. The log concentration-response data was plotted and best-fit sigmoidal curves were determined using SlideWrite Plus Version 3.0. The coefficient of determination for the curves ranged from 0.90 to 0.99 and concentration which inhibited cell proliferation by 50% (IC_{50}) was determined using the equation for the best-fit curve.

4.3.10 Slot Blot

For slot blot experiments, cells were treated as outlined in section 4.2.5 and, at specified time points, a volume of tissue culture medium containing 1 x 10^5 cells was removed. Samples were then washed three times in cold PBS, and frozen at -20°C. In some experiments, samples were also subjected to three washes in 0.8 M NaCl, a one hour proteinase K digestion (15 mg/mL in PBS, on ice) followed by two washes in PBS before freezing. At a later time, samples were thawed, and an NaOH/EDTA solution was added to give a final concentration of 0.4 M NaOH and 10 mM EDTA, and the samples were boiled for 10 minutes. The samples were blotted onto a Zeta-Probe® GT membrane (BioRad) using a Bio-Dot SF slot blot apparatus (BioRad), following the protocol outlined by the manufacturer. After blotting and rinsing, the membrane was dried at 80°C for 2 hours and stored at room temperature. Membranes were probed using a gel purified 18-mer phosphodiester ^{32}P-ODN which was complementary to the *MDR1* asODN (sODN). The probe was prepared using [γ-^{32}P]ATP (3000 Ci/mmol, DNA Core Services, University of Alberta) and T4 polynucleotide kinase (Gibco BRL, Burlington, ON), following the protocol outlined by the manufacturer. Free [γ-^{32}P]ATP was removed by separation on a BioSpin P-6 spin column (BioRad). The hybridization was carried out using ExpressHyb (Clontech) following the protocol outlined by the manufacturer, developed overnight, and analysed the next day using a Model GS-250 Molecular Imager™ System (BioRad, Mississauga, ON).

Statistical analyses were carried out using Systat 5.02 for Windows (Systat Inc., Evanston, IL). For the slot-blot assay, a one-way analysis of variance was employed, followed by pair-wise comparisons using the Tukey-Kramer method. For all other experiments, Student t-tests were performed between the asODN and the sODN treated groups.

4.4 RESULTS

4.4.1 Characterization of the Namalwa/MDR$_{40}$ cell line

4.4.1.1 Northern analysis

In order to confirm that the Namalwa/MDR$_{40}$ cell line expresses the *MDR1* gene, Northern analysis and flow cytometry were carried out to evaluate levels of *MDR1* RNA and P-gp, respectively. Figure 4.1 is a phosphorimage of a Northern blot comparing the relative expression of *MDR1* RNA between CEM/VLB$_{100}$, Namalwa/MDR$_{100}$, Namalwa/MDR$_{40}$ and Namalwa/parent cells. The first observation from Figure 4.1 is the striking difference between the levels of *MDR1* among the different cell lines. There was no signal for *MDR1* in the lane loaded with RNA from the Namalwa/parent cells, while the CEM/VLB$_{100}$ cells and the Namalwa/MDR$_{100}$ cells demonstrate a significant signal for *MDR1*. For the exposure time used for this image, there appears to be little signal from the Namalwa/MDR$_{40}$ cells; however, longer exposure times demonstrated a definite signal in this lane, with no detectable signal from the Namalwa/parent cell RNA.

The next observation is the pattern of *MDR1* signal observed in the Namalwa/MDR$_{40}$ and Namalwa/MDR$_{100}$ cells compared to the CEM/VLB$_{100}$ cells. The CEM/VLB$_{100}$ cells express an *MDR1* RNA with a molecular weight of approximately 4.5 kb (close to the 28S rRNA) which is consistent with the known size of the *MDR1* RNA (182) and previously published results using this cell line (186). Both Namalwa/MDR lines demonstrated a signal for an RNA with the same molecular weight; however, there

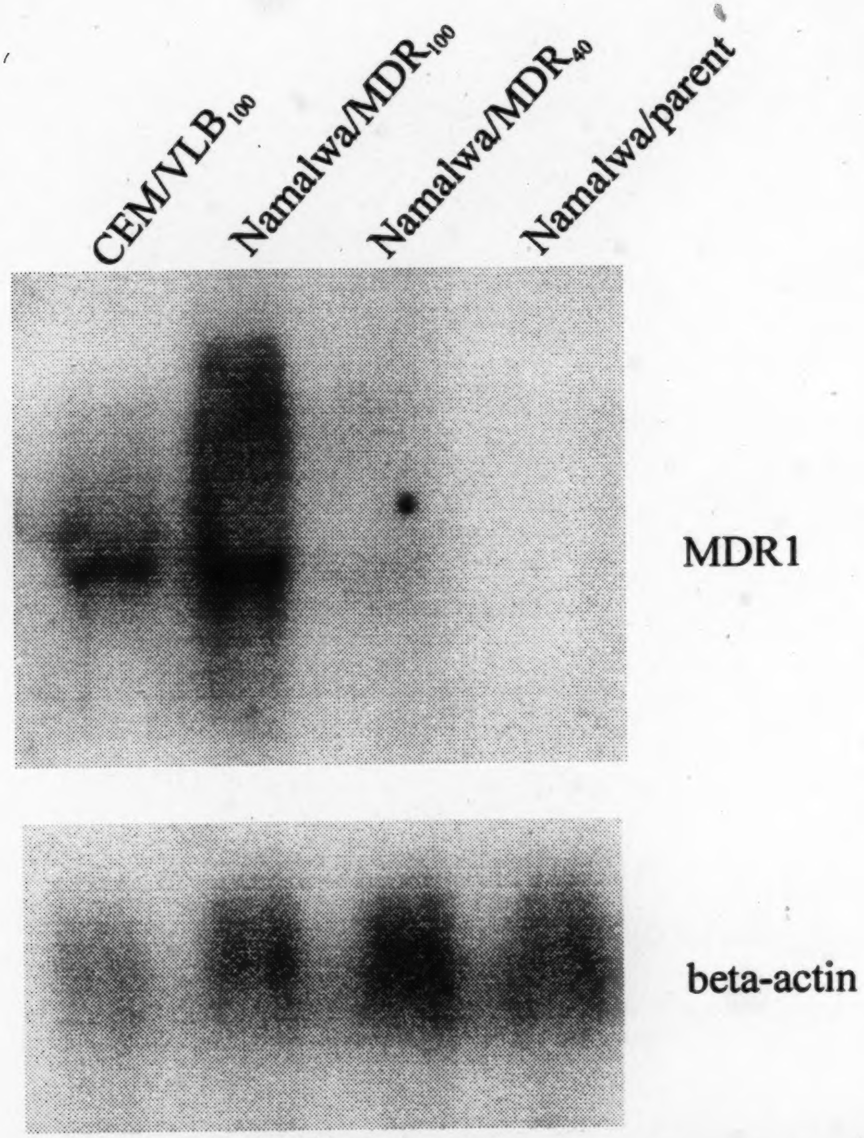

CEM/VLB100
Namalwa/MDR100
Namalwa/MDR40
Namalwa/parent
MDR1
beta-actin

Figure 4.1: Northern analysis of *MDR1* expression in four different cell lines. Total cell RNA (17 μg/lane) was electrophoresed on a denaturing agarose gel and then transferred to a nylon membrane. The membrane was probed for *MDR1* using a ^{32}P-labelled probe, analysed using a phosphorimager, and then stripped and re-probed using a ^{32}P-labelled β-actin probe.

was also staining of a higher molecular weight message which sometimes gave a stronger signal than the 4.5 kb band. Other cell lines transfected with recombinant *MDR1* copy DNA (cDNA) demonstrate similar patterns for Northern analyses (202) but still express P-gp and display the MDR phenotype. The larger transcript probably contains parts of the long-terminal repeats from the Harvey murine sarcoma virus which are adjacent to the *MDR1* cDNA in pHaMDR1/A.

Since the Namalwa/MDR$_{40}$ cells demonstrated the lowest signal for *MDR1* mRNA compared to the other MDR cell lines, it probably represents a more clinically relevant level of resistance and therefore I decided that it would be the most appropriate line to use for antisense studies.

4.4.1.2 Analysis of cell surface P-glycoprotein expression

Figure 4.2 illustrates the flow cytometry results for the P-gp expression in the Namalwa/MDR$_{40}$ cells. Since my goal will be to use αCD19-targeted liposomes to deliver *MDR1* asODN to these cells, cell surface expression of CD19 was also determined. Figure 4.2A indicates that the Namalwa/parent cells express CD19 as evident by the 2.5 log shift in fluorescence of cells stained with αCD19 mAb; however, cells stained with α-P-gp mAb (MRK16) demonstrated only background fluorescence. Namalwa/MDR$_{40}$ cells stained for CD19 to a similar degree as the Namalwa/parent cells; however, there was also significant staining for P-gp (2-log shift in fluorescence). These results confirm that

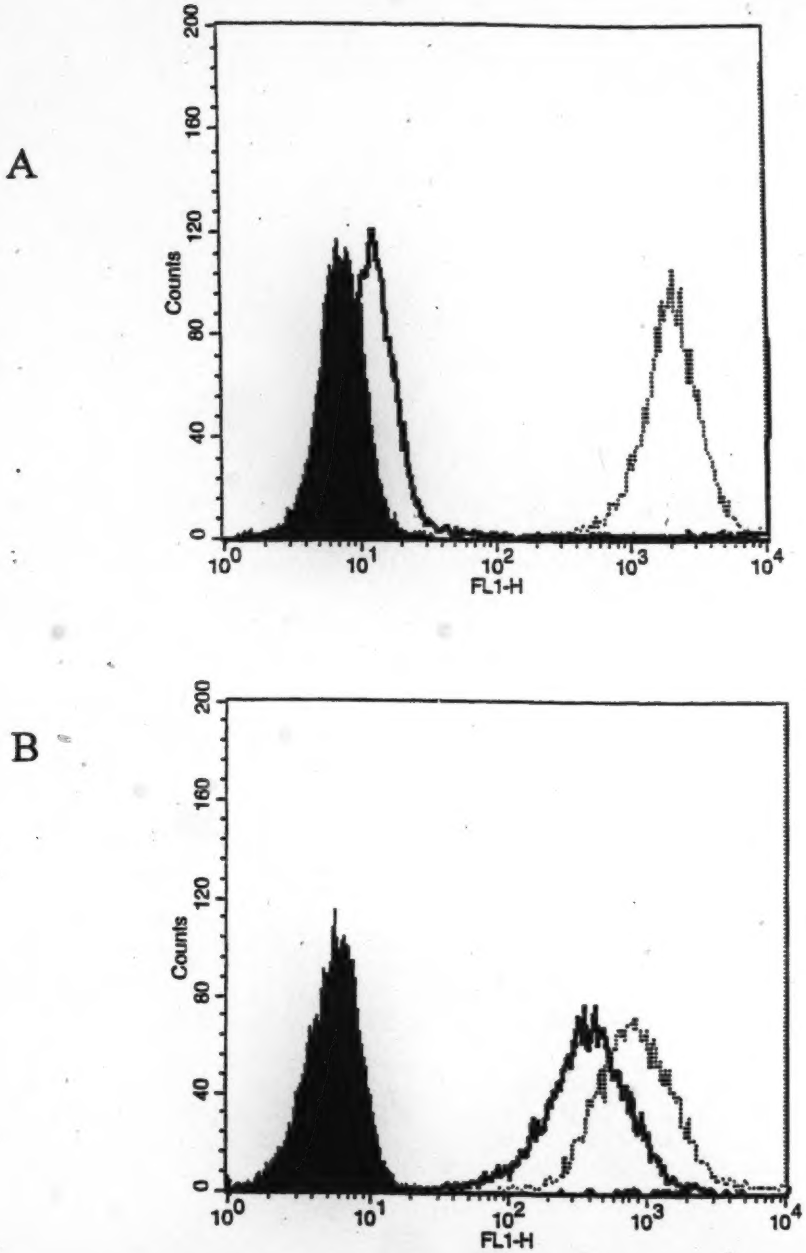


Figure 4.2: Flow cytometry analysis of Namalwa/parent (A) and Namalwa/MDR$_{40}$ (B) cell lines. The filled histogram represents the intrinsic fluorescence of the cells, the dark lines represents staining of cells for P-gp using MRK-16 mAb, and the light lines represent cells stained for CD19 using αCD19 mAb.

Namalwa/MDR$_{40}$ cells express P-gp, as well as CD19, which will be important for specific targeting of asODN to these cells.

4.4.1.3 Analysis of P-glycoprotein activity

The calcein accumulation assay provides a simple and rapid determination of P-gp activity. Calcein-AM is a non-fluorescent, membrane permeable derivative of calcein which has been shown to be a substrate for P-gp (203). Once it diffuses through the cell membrane, intracellular esterases cleave the acetoxymethyl ester, releasing fluorescent and hydrophilic calcein. Cells expressing P-gp will pump the non-fluorescent calcein-AM from the membrane before it can be cleaved by intracellular esterases, while cells not expressing P-gp will accumulate fluorescent calcein. Figure 4.3 illustrates the difference in fluorescence between the Namalwa/parent cells and the Namalwa/MDR$_{40}$ cells. Following a 15 minute incubation with calcein-AM, there was an 18-fold difference in fluorescence between the parent and MDR cell lines. These results are consistent with the cell-surface expression of P-gp in the Namalwa/MDR$_{40}$ cells.

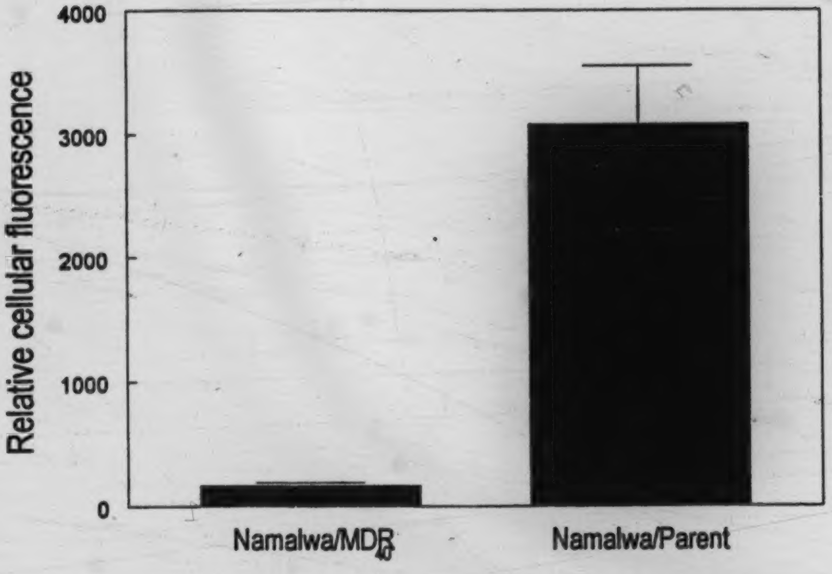


Figure 4.3: Characterization of P-gp activity using the calcein accumulation assay. Cells were incubated for 15 minutes with calcein-AM, followed by 3 washes with cold serum-containing medium. Calcein fluorescence was determined using a 96-well fluorescent plate reader, and each bar represents the mean ± standard deviation from 5 different samples.

4.4.1.4 Doxorubicin sensitivity

In order to confirm that the expression and activity of P-gp in Namalwa/MDR$_{40}$ cells resulted in a measurable change in the sensitivity to a chemotherapeutic agent, the IC$_{50}$ for doxorubicin was determined and compared to Namalwa/parent cells. Figure 4.4 shows the dose-response curves for both cell lines over a range of doxorubicin concentrations. In this example, the IC$_{50}$ was approximately 10-fold higher for the MDR$_{40}$ cells compared with the parent cells, and when the experiment was repeated three times, the IC$_{50}$ for the MDR$_{40}$ cells was 8.77 μM ± 0.19 (S.D.) and the IC$_{50}$ for the parent cells was 0.81 μg/mL ± 0.10.

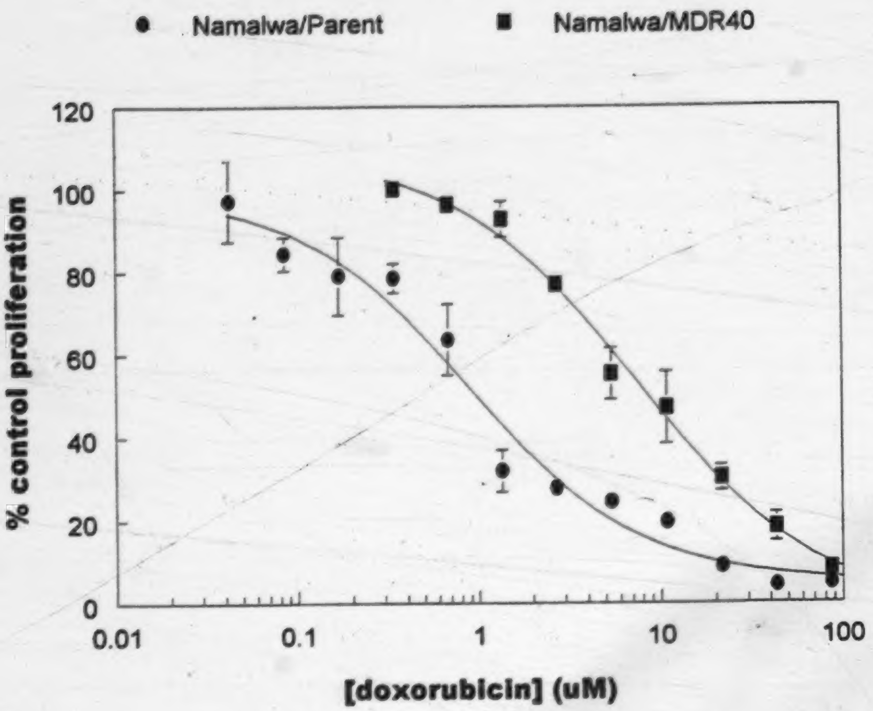


Figure 4.4: Cytotoxicity of doxorubicin in Namalwa/parent and Namalwa/MDR$_{40}$ cells. Cells were incubated for one hour at 37°C in the presence of different concentrations of doxorubicin, washed three times in serum-containing media, and incubated for 47 hours at 37°C. Cell proliferation was measured using the MTT assay as outlined in the Methods section 4.2.9. Each point represents the mean of 3-5 samples ± standard deviation.

4.4.2 Cellular association of liposomal asODN

The slot blot-hybridization method represents a simple method to determine the levels of asODN within biological fluids or associated with cells (165). The method ensures that only intact and functional ODN will be measured and offers advantages over methods which use radiotracers alone or in combination with chromatographic methods. A standard curve can be created by adding known amounts of asODN to cells/tissues and then blotting onto the membrane along with the test samples. Figure 4.5 illustrates the phosphorimage of a membrane blotted with standards, along with the densitometric analysis and the standard curve which was generated. This demonstrates that the assay is sensitive down to approximately 6 ng and is linear up to at least 200 ng asODN. The equation of this line was used to calculate the amount of asODN per 1×10^5 cells by integrating the area under the densitometric curve for test samples.

The results from an experiment comparing the delivery of asODN using CCL, αCD19-CCL, LipofectAMINE®, or free, are illustrated in Figure 4.6. Incubation of cells with free asODN resulted in the lowest levels of cell-associated asODN; however, cell uptake could be significantly ($p<0.01$, one-way ANOVA) increased by using LipofectAMINE® as a delivery vehicle. When CCL were used to deliver asODN, the amount of cell-associated asODN increased only slightly over that seen for free asODN and this increase was not significant ($p>0.05$, one-way ANOVA); however, asODN delivery was improved by attaching αCD19 mAb onto the surface of CCL. There was a

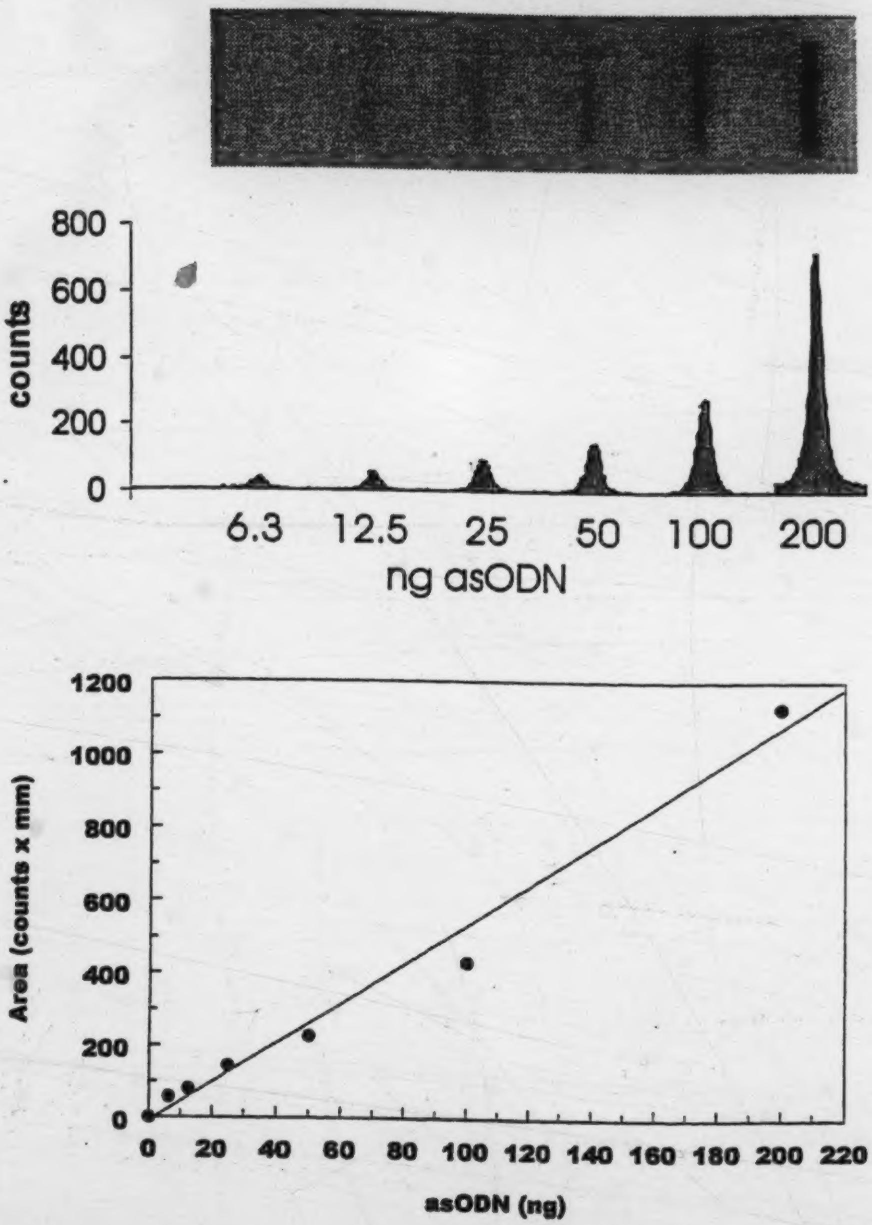


Figure 4.5: Phosphorimage and standard curve for slot-blot assay. Different quantities of *MDR1* asODN (200 - 6.25 ng) were added to 1 x 10^5 Namalwa/MDR$_{40}$ cells, boiled in 0.4 M NaOH, 10 mM EDTA and blotted onto a nylon membrane along with test samples and hybridized with ^{32}P-sODN probe. The areas under the densitometric curves for each amount of asODN were then used to construct a standard curve.

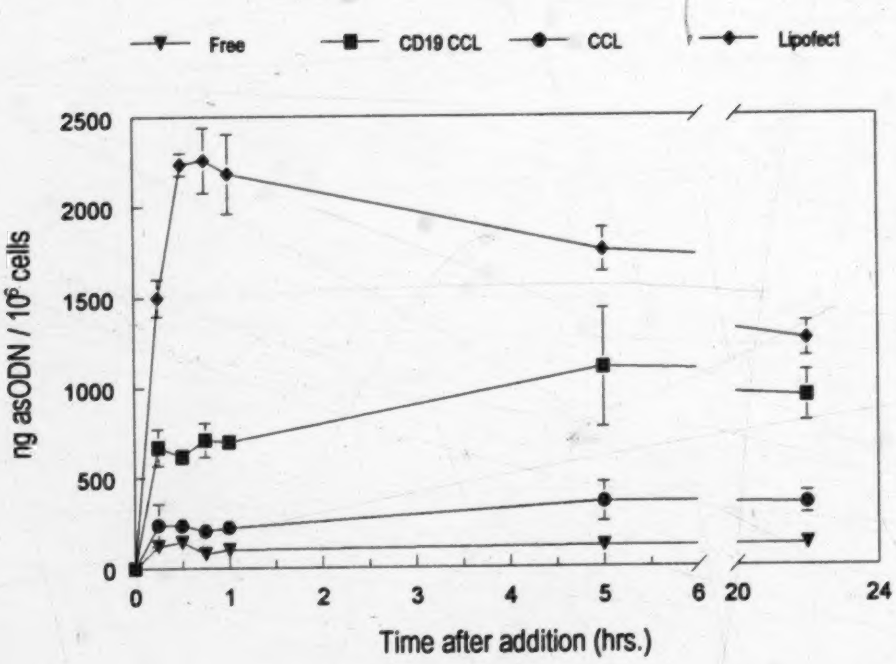


Figure 4.6: Cellular association of *MDR1* [S]-asODN using different delivery vehicles. Values were determined using the slot-blot assay. Each point represents the mean ± standard deviation of 3 different samples.

significant ($p<0.01$, one-way ANOVA) increase (up to 3.5-fold) in cell uptake compared to non-targeted CCL at all time points. By 22 hours, delivery of asODN by αCD19-CCL approached the values obtained using LipofectAMINE®.

The data in Figure 4.6 do not discriminate between asODN which is associated with the cell membrane, and that which is internalized. Therefore another experiment was performed in which aliquots of cells from each group were rinsed in 0.8 M NaCl, and subjected to protease treatment, in order to remove asODN associated with the outside of cells and compare them to aliquots which were simply washed with PBS. The data in Table 4.1 indicate that, following 48 hours incubation, 79% of the cell-associated asODN could be removed by the NaCl/protease treatment if the asODN was delivered using CCL, suggesting that 21% of the asODN has been taken up by the cells. When αCD19-CCL were used to deliver asODN, only 58% of cell-associated asODN could be removed, suggesting that almost one-half of the asODN has been taken up. LipofectAMINE® mediated delivery resulted approximately two-thirds of asODN apparently internalized, which is consistent with the fusogenic nature of this positively charged carrier. For free asODN, 32% of the cell-associated asODN was resistant to NaCl/protease, suggesting that about one third of the asODN was taken up. It should also be noted that the data in Table 4.1 (ng asODN / 10^6 cells without NaCl/protease) does not correlate exactly with the Figure 4.6 because of the time points measured (48 hours in Table 4.1 vs. 15 minutes to 22 hours in Figure 4.6) and because they represent results from two different experiments. Further discussion of these results follows in section 5.0.

Table 4.1: Uptake of asODN by Namalwa/MDR$_{40}$ cells after 48 hours incubation with different carriers. Three aliquots of cells from each group were washed 3 times in PBS (-protease) and three different aliquots were subjected to the NaCl/protease treatment (+protease). Each value represents the mean of three different samples ± standard deviation.

Carrier	**ng asODN / 10^6 cells**		**Protease resistant**
	- protease	+ protease	**%**
CCL	415 ± 19.9	88 ± 9.0	21
αCD19-CCL	657 ± 11.2	273 ± 23.6	42
LipofectAMINE®	357 ± 55.5	227 ± 22.9	64

4.4.3 *MDR1* RNA levels in cells treated with asODN

Phosphorothioate asODN are thought to act primarily by an RNase H-dependent mechanism, and therefore asODN treatment should result in a decrease in *MDR1* mRNA levels. Cells were collected after 24 hours treatment (as outlined in section 4.2.5) and total RNA was isolated and analysed by Northern blot. Figure 4.7 is a phosphorimage of a membrane which was hybridized with ^{32}P-*MDR1* probe, stripped and then re-probed with a ^{32}P-β-actin probe. As discussed earlier, hybridization with ^{32}P-*MDR1* probe results in two visible bands for the Namalwa/MDR40 cells, and in this case, the high molecular weight RNA gave a stronger signal than the typical 4.5 kb *MDR1* RNA. Similar results were obtained when RNA was collected and analysed following 48 hours of incubation with asODN (data not shown). In order to quantitate the signal, densitometry was performed on an area of each lane large enough to contain both bands, and the entire signal was integrated. The results in Figure 4.7 suggest that there are subtle differences in the RNA levels, and in this example, treatment with α-CD19-CCL[asODN], CCL[asODN], and 20 μM free asODN resulted in decreases relative to their respective sODN controls. Table 4.2 gives the averages of the Northern analyses from three different experiments and these results indicate that the only group which demonstrated a significant ($p<0.05$, two-tailed t-test) decrease in *MDR1* RNA relative to the sODN control was the high concentration (20 μM) of free asODN. In addition, treatment with a low concentration (1 μM) of free asODN did not decrease *MDR1* RNA levels ($n = 1$).

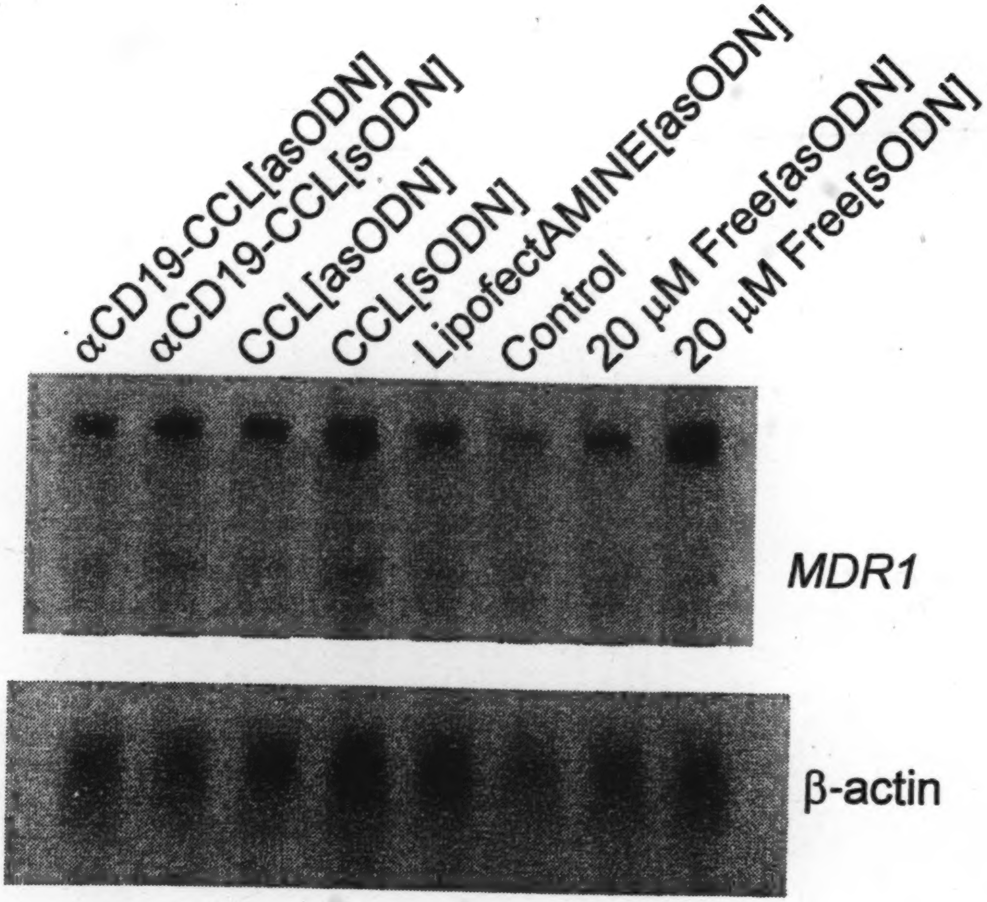

αCD19-CCL[asODN]
αCD19-CCL[sODN]
CCL[asODN]
CCL[sODN]
LipofectAMINE[asODN]
Control
20 μM Free[asODN]
20 μM Free[sODN]
MDR1
β-actin

Figure 4.7: Northern analysis of Namalwa/MDR$_{40}$ cells treated for 24 hours with *MDR1* asODN/sODN delivered using different delivery vehicles. Total cell RNA (17 μg/lane) was electrophoresed in a denaturing agarose gel and then blotted onto a nylon membrane overnight. The membrane was hybridized with a ^{32}P-*MDR1* probe, visualized on a phosphorimager, and then stripped and reprobed for β-actin.

Table 4.2: Summary of Northern blot analyses. Effect of different asODN treatments on the expression of *MDR1* relative to the sODN control. The relative *MDR1* message (*MDR1* signal/β-actin signal) was normalized to the sODN control for each group. Each point represents the mean ± standard deviation of three experiments.

Carrier	**[ODN] μM**	***MDR1* Message (% of sODN control)**
αCD19-CCL[asODN]	0.7	112 ± 23.1
CCL[asODN]	0.7	104 ± 14.0
LipofectAMINE asODN	1.0	96 ± 60.0
Free asODN	20	67 ± 16.1

4.4.4 Effect of *MDR1* asODN treatments on P-glycoprotein activity

The calcein accumulation assay was used to evaluate the activity of P-gp following treatment with asODN/sODN using different carriers. An increase in fluorescence caused by *MDR1* asODN treatment would suggest a decrease in P-gp levels due to antisense activity. Cells were incubated for 72 hours in the presence of *MDR1* asODN or sODN delivered using α-CD19-CCL, CCL, LipofectAMINE®, or free, and Figure 4.8 illustrates the results of the calcein accumulation assay. In each case, results are expressed as a percentage of control cell fluorescence, with the control being sODN delivered using the same vehicle. Treatment with LipofectAMINE®[asODN], CCL[asODN] and 13 μM free asODN did not result in significant increases in fluorescence relative to their sODN controls. In contrast, treatment with anti-CD19-CCL[asODN] resulted in a 65% increase in cellular fluorescence, compared to the anti-CD19-CCL[sODN] control ($p < 0.01$, two-tailed t-test). These sequence-specific results suggest that the expression of P-gp is being inhibited, resulting in a net decrease in P-gp activity.

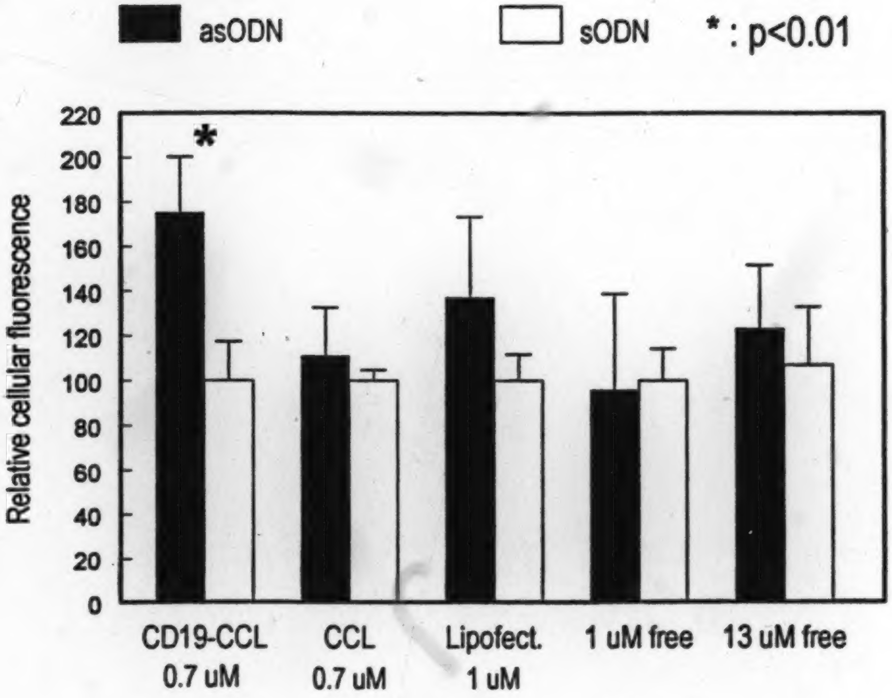


Figure 4.8: Calcein accumulation assay for Namalwa/MDR$_{40}$ cells following 72 hours of treatment with *MDR1* asODN/sODN delivered using different delivery vehicles. Each bar represents the mean from 5-10 different samples ± standard deviation.

4.4.5 Effect of asODN treatments on doxorubicin cytotoxicity

The ultimate goal of antisense inhibition of the *MDR1* gene is to decrease cellular levels of P-gp, and therefore increase the sensitivity of the cells to chemotherapeutic agents, such as doxorubicin. Therefore, cells were treated for 72 hours with the different asODN/sODN delivery vehicles, and then tested for their sensitivity to doxorubicin. A decrease in the expression of *MDR1* resulting in a decrease in the amount of P-gp, should result in a decrease in the IC_{50} for doxorubicin. Table 4.3 gives the average IC_{50} values from three such experiments; statistical analyses of these data indicate that there are no significant decreases in IC_{50} values for any of the asODN treatment groups relative to their sODN controls.

Table 4.3: Effect of asODN / sODN treatments on the doxorubicin sensitivity of Namalwa/MDR$_{40}$ cells. Each point represents the mean ± standard deviation from three experiments, except for the 20 μM free groups which represent the IC$_{50}$ from one experiment.

Carrier	**[ODN] μM**	**Doxorubicin 1 hour IC$_{50}$ (μM)**	
		asODN	**sODN**
αCD19-CCL	0.7	10.3 ± 3.49	6.9 ± 4.31
CCL	0.7	8.9 ± 0.93	6.2 ± 1.68
Free	1	12.5 ± 4.29	7.5 ± 0.93
Free	20	11.4	13.9
Control	8.8 ± 0.19		
Namalwa/Parent	0.8 ± 0.10		

4.5 DISCUSSION

The most significant and interesting results from this work come from the slot-blot experiments which measured the amount of cell-associated asODN. At early time points (up to 22 hours) LipofectAMINE® was the most efficient formulation for delivering asODN and resulted in greater than a 20-fold increase in the amount of cell-associated asODN compared to free asODN. This result was not unexpected since cationic liposomes have been shown in many instances to increase the cellular delivery and activity of asODN (94, 204). Lipoplexes, which carry an excess positive charge, mediate the intracellular delivery of asODN through electrostatic interactions with the cell membrane resulting in endocytosis, followed by release into the cytoplasm (97, 101). As described in the Introduction, the mechanism(s) by which free phosphorothioate asODN are taken-up by cells is less clear, possibly involving fluid-phase or adsorptive endocytosis, a receptor-mediated mechanism, or a combination of all three (28, 176-179).

As outlined in the Introduction, the CCL formulation is made at a 1:1 charge ratio (+ / -) with the asODN, and therefore there is no excess positive charge to interact with cell membranes. In addition, CCL contain PEG-DSPE which will further reduce their interaction with cells. Therefore, it is not surprising that the amount of cell-associated asODN following incubation with CCL was only slightly higher than for free asODN, and significantly less than for LipofectAMINE® delivery. The most important observation from Figure 4.6 is that by attaching a mAb to the surface of CCL, which recognizes the target cells, the amount of cell-associated asODN is significantly increased compared to non-targeted CCL. At early time points, the amount of asODN delivered to cells by αCD19-CCL was much lower compared to LipofectAMINE®; however, from 5 to 22 hours the difference was only 33% and 25% respectively.

The amount of cell-associated asODN remains relatively constant or increases slightly for cells treated with αCD19-CCL, CCL or free asODN, but decreases from 1 hour to 22 hours for cells treated with LipofectAMINE®. The reason for this decrease is not absolutely clear, but may reflect a dilution effect due to the proliferation of cells. All experiments were carried out in serum-containing medium, since previous experiments (data not shown) indicated that there was no difference in the delivery or activity of the CCL formulation in the presence of serum. LipofectAMINE®, on the other hand, is known to be much less active in serum-containing medium, and therefore after the first hour when cell uptake peaks, the complexes are no longer active. As the cells proliferate over the course of 22 hours (doubling time approx. 24 hours), the cell-associated asODN is diluted among the dividing cells. The αCD19-CCL, CCL and free asODN are all relatively stable in serum-containing medium, and therefore cells may continually accumulate asODN as they proliferate, resulting in relatively constant levels. An alternative hypothesis is that asODN delivered using LipofectAMINE® are internalized to a greater extent, resulting in degradation of asODN in lysosomes; however, phosphorothioate asODN are resistant to nucleases.

The data in Table 4.1 represent cellular asODN levels following 48 hours incubation and were generated from a separate experiment than the data in Figure 4.6. By 48 hours, αCD19-CCL resulted in the highest levels of cell-associated asODN compared to LipofectAMINE® or CCL, with levels consistent with the data in Figure 4.6. The amount of asODN delivered using LipofectAMINE® has continued to decrease, while the amount of asODN delivered using αCD19-CCL and CCL has remained relatively constant. The data in Table 4.1 also suggest that a greater proportion of the asODN delivered using LipofectAMINE® is internalized, since this group demonstrated the highest proportion of asODN resistant to the NaCl/protease salt wash. Consistent with previous studies which indicate that αCD19-targeted liposomes are internalized (88, 197), a greater proportion of asODN delivered using αCD19-CCL was resistant to the NaCl/protease salt wash, compared to the non-targeted CCL.

Unfortunately the experiments described in this chapter do not indicate that the CCL formulations are effective at delivering an active asODN. This is probably due to the antisense model, rather a lack of efficacy of the CCL formulations, since treatment with cationic lipoplexes (LipofectAMINE®), which have been shown to be active in several other instances, were also not able to produce an antisense effect. Targeting *MDR1* in Namalwa/MDR$_{40}$ cells with asODN appeared to be a good model in which to evaluate different liposomal asODN formulations. The degree of resistance to doxorubicin observed in these cells is more clinically relevant than other MDR cell lines such as the

CEM/VLB$_{100}$ cells which are greater than 200-fold resistant (198). In addition, the Namalwa/MDR$_{40}$ cells express CD19 which internalizes upon binding by αCD19-targeted liposomes (88, 197), allowing us to study the efficacy of targeted liposomes containing asODN.

However, an odd characteristic of the Namalwa/MDR$_{40}$ cells is that they have a very low level of *MDR1* mRNA which is barely discernable next to other MDR cell lines (Figure 4.1), while flow cytometry results indicate that the cells stained quite readily for P-gp. This could indicate that the protein is very stable with a long metabolic half-life. In fact the half-life of P-gp in cultured cells has been reported to range from 14 to 18 hours (205, 206), but the potential for P-gp to have a longer half-life cannot be ruled out since at least one study suggests that the half-life can be as long as 48 hours (207). Resistance in the Namalwa/MDR$_{40}$ cells could come from enhanced stability of P-gp as well as the overexpression of *MDR1*. If this is the case, it would be very difficult to significantly decrease the P-gp levels and therefore resistance by inhibiting translation of *MDR1* mRNA.

The results in Table 4.3 indicate that none of the asODN treatments had a significant effect on the sensitivity of the cells to doxorubicin, suggesting that P-gp levels were not significantly decreased by asODN treatment. However, the response of cells to a chemotherapeutic agent such as doxorubicin may be quite complex and may have secondary effects on the expression of *MDR1*. Chemotherapeutic agents have been shown to induce specific gene expression (208) and activate *MDR1* (185, 186). In addition, I have demonstrated that Namalwa/MDR$_{40}$ cells express *MDR1* and cell surface P-gp, however, there may be other mechanisms of resistance which decrease the sensitivity to doxorubicin and would not be affected by *MDR1* asODN treatment.

The calcein accumulation assay is a more direct measure of P-gp activity since it measures the ability of cells to extrude calcein-AM over a very short time course (15 minutes) compared to the doxorubicin cytotoxicity assay which involves a one hour exposure followed by a 47 hour incubation. Also, since the calcein accumulation assay is not affected by cell proliferation, and is less likely to be affected by secondary effects of the drug, it should be more sensitive to small changes in P-gp levels. The results in Figure 4.7 indicate that αCD19-CCL asODN treatment decreased P-gp activity, suggesting that P-gp levels have been decreased. While the increase (1.8-fold) in fluorescence was significant ($p < 0.01$) relative to the sODN control, cellular fluorescence did not approach values observed in the Namalwa/parent cells which do not express *MDR1* (18-fold more fluorescent than Namalwa/MDR$_{40}$ cells). Therefore, the potential decrease in P-gp levels is quite small and the doxorubicin sensitivity assays suggest that it would not be therapeutically significant.

Phosphorothioate asODNs inhibit translation via RNase H-dependent mRNA degradation (13, 19). The asODN-mRNA hybrid is recognized by RNase H and degrades the mRNA strand, leaving the asODN intact. Therefore the *MDR1* asODN treatments should have resulted in decreases in *MDR1* mRNA; however, the results in Table 4.2 indicate that the only asODN treatment which resulted in decreases in *MDR1* mRNA relative to the sODN control was when the cells were treated with 20 µM free asODN. This contrasts with the results obtained for the calcein accumulation assay in which αCD19-CCL[asODN] treatment apparently decreased P-gp levels. The apparent decrease in P-gp activity was not coupled with a decrease in *MDR1* mRNA, suggesting that if the observed decrease in activity is real, it is not a result of antisense inhibition mediated by RNase H. The other question which arises is why was there a decrease in *MDR1* mRNA in cells treated with free asODN, but there was no change in P-gp activity or doxorubicin sensitivity? It is possible that the 33% decrease in *MDR1* mRNA was not enough to cause a significant change in the amount of P-gp at the cell surface.

Regardless of the stability or half-life of P-gp noted above, the fact that there were no remarkable decreases in *MDR1* mRNA observed by Northern analysis suggest that the asODN was not active, since phosphorothioate asODN are known to activate RNase H which would result in reduced levels of mRNA.

The strategy of using asODN targeted to the *MDR1* gene in order to reverse multidrug-resistance in cancer cells has been examined before. AsODN targeted to the *MDR1* gene has been shown to decrease *MDR1* mRNA and P-gp levels resulting in partial reversal of multidrug-resistance *in vitro* (132, 150, 191-196). Most of these studies demonstrated decreases in *MDR1* mRNA, and/or P-gp levels, and/or increased sensitivity of cells treated for 24 to 72 hours with asODN (191-194, 196). The study by Alahari *et al.* (194) clearly demonstrated that at 24 hours maximum reduction in *MDR1* mRNA was observed and levels were not further reduced after 48 or 72 hours incubation with asODN. This suggests that the incubation times used in my studies should have been long enough to at least see a change in the cellular levels of *MDR1* mRNA. The other studies also suggest that the 72 hour incubations used in my studies should have been long enough to observe increases in dye accumulation and drug sensitivity. Furthermore, the *MDR1* antisense sequence used in my studies was the same sequence as that used by Cucco and Calabretta (150) and was targeted to the initiation codon, which has been shown to be an appropriate target in other studies. In fact, all of the studies which demonstrated inhibition of *MDR1* using asODN targeted sequences which were overlapping, or very close to, the initiation codon.

The results described in this chapter suggest that treatment of Namalwa/MDR_{40} cells with *MDR1* phosphorothioate asODN does not result in a profound inhibition of resistance. The ultimate goal of this strategy would be to inhibit the expression of *MDR1* and subsequently increase the sensitivity of these cells to chemotherapeutic agents. The targeted and non-targeted CCL would be suitable carriers with which to test the effectiveness of liposomal versus free asODN in an animal model. Unfortunately the negative results did not warrant *in vivo* testing at this time. The inability to produce a significant antisense effect is not surprising, since antisense technology has been riddled with complications and non-reproducible results (reviewed in (26, 209-211). In spite of these results, we have demonstrated that the CCL formulation can be targeted by attaching a mAb resulting in increased uptake of asODN compared to non-targeted asODN. In another model, our collaborators have demonstrated that targeted-CCL can deliver an active c-myb asODN to target cells resulting in decreases in c-myb protein and inhibition of cell proliferation (submitted for publication). We can conclude that targeted-CCL can increase the delivery of asODN compared to non-targeted-CCL and in a suitable experimental model this can result in measurable inhibition of gene expression.

CHAPTER 5

Summarizing discussion, conclusions and future directions

Over the past 20 years the strategy of inhibiting gene expression through antisense mechanisms has developed from a tool used to examine the kinetics of mRNA translation, and matching cDNA with mRNA and protein, to a therapeutic tool used to inhibit the expression of disease-related genes. New drugs or novel therapeutic strategies, such as asODN, often attract a great deal of hype and promise to revolutionize medicine, but this is often before good basic science has been done. The reality rarely lives up to the hype, and a great deal of controversy has surrounded the antisense field (26, 209-211). In spite of the disappointments in clinical applications of asODN, there is at least one bright spot. The very first approved antisense drug to receive approval by the United States Food and Drug Administration, Vitravene^{TM}, developed by ISIS Pharmaceuticals, is a phosphorothioate asODN targeted to cytomegalovirus (CMV) and administered by intra ocular injection for the treatment of CMV retinitis in AIDS patients. ISIS has other antisense drugs in various stages of development, and one that deserves mention is ISIS 2302, which is a phosphorothioate asODN targeted to intracellular adhesion molecule 1 (ICAM-1). ICAM-1 plays a role in the accumulation and extravasation of leukocytes into areas of inflammation, and therefore ISIS 2302 has demonstrated efficacy in the treatment of Crohn's disease (currently in Phase III clinical trial), ulcerative colitis, prevention of kidney transplant rejection, rheumatoid arthritis, and psoriasis (currently in Phase II clinical trials). Both ISIS 2302 and Vitravene^{TM} are administered without a carrier.

Throughout this period of development, cationic liposomes have played an important role in the preclinical testing and evaluation of asODN. As discussed throughout this thesis, cationic liposomes are often an absolute requirement for antisense activity *in vitro*. However, there is very little evidence supporting the usefulness of liposomal carriers for asODN *in vivo*, and there is a considerable amount of experimental evidence which indicates that free asODN are active *in vivo*. Very little is known about why free asODN may be active *in vivo*, but inactive *in vitro*. It may involve a phenotypic difference between cultured cell lines and cells within in an intact organ *in vivo*. This still doesn't answer the question of why liposomal carriers have not proved useful *in vivo*. Throughout this thesis, I have made the argument that liposomal asODN carriers have not been optimized for use *in vivo*. The examples from the literature of liposomal asODN indicate that circulation times are very short and the liver accumulates the majority of the dose. In order to reach significant concentrations outside of the liver, different formulations will have to be used.

It could be argued that a large proportion of antisense research is being carried out by, or sponsored by, private industry. As drug products, asODN would be more difficult to manufacture, store and use if they are formulated in a liposomal carrier. Put another way, formulating asODN in a carrier may ultimately be more expensive, and therefore if they are active in free form, this would be the easiest and least expensive way to produce them. Companies like ISIS are no doubt conducting research into delivery systems for asODN; however, their focus is probably to find ways of making free asODN work. An exception to this generalization may be INEX Pharmaceuticals (Burnaby, BC). INEX has taken special interest in developing lipid-based carriers for asODN which may be tested in clinical trials in the near future.

It is difficult to estimate the number of therapeutic or *in vivo* applications which have not worked in pre-clinical testing, since negative results are rarely published. Could a liposomal carrier have made a difference? The antisense field has attracted more controversy than any other with regard to irreproducible results and questionable secondary effects. A comment from one of the reviewers who reviewed the contents for Chapter 3 was that "Antisense technology has been controversial, to say the least". In light of the uncertainty in the antisense field, I would suggest that it is too early to draw any firm conclusions regarding the potential of liposomal, or other, carriers for asODN.

In the tens to hundreds of therapeutic applications which have failed, we should evaluate what the problems were. If a link can be drawn between a disease and the expression of a gene, then one should be able to design an asODN which will hybridize the target mRNA by utilizing gene bank data or sequencing methods. Unfortunately it is not that simple, and the reasons for the lack of efficacy of some sequences is not well understood. The method for choosing an antisense sequence is empirical; 10 or more different antisense sequences for the same gene are synthesized and screened for activity. It has been suggested that only about 10% of all sequences tested demonstrate significant activity (26) and methods have only recently been developed which attempt to predict which antisense sequences may be most active (212). The approach used for the experiments in this thesis was to review the literature and use a sequence which has previously demonstrated activity. As described in Chapter 4, the sequence I used was the same as that used by Cucco and Calabretta and was targeted to the *MDR1* initiation codon (150). This region of the *MDR1* mRNA was also targeted in several other studies as described in Chapter 4.

Once an active sequence has been found, the next problem becomes a question of delivery. Can we get enough drug (asODN) to the receptor (mRNA) to result in inactivation of a large proportion of the target mRNA? This has been one of the most frequently cited hurdles to successful antisense therapy (14). For the single asODN drug which has been approved for use in humans, delivery is not a problem. Vitravene™, which is used to treat CMV retinitis in AIDS patients, is administered by local, intravitreal injection. The vitreous body, in the posterior globe of the eye, may be the most effective slow-release gel that Nature could have created. It is a highly viscous gel through which asODN may slowly diffuse, providing a prolonged exposure to the area of infection, the retina. This method of delivery by-passes the potential problems encountered upon systemic administration. Therapeutic applications in which asODN have failed may have worked if increased levels of asODN were achieved in the target tissue. The potential for a targeted liposomal carrier must be considered.

In the Introduction (Chapter 1), I summarized the historical development of liposomes as drug carriers. The knowledge gained over the past 25 years should be applied to the current goal of developing a suitable liposomal carrier for asODN. By highlighting the problems with liposomes, and how these problems were overcome, I attempted to provide an adequate background needed to judge the potential suitability of a given formulation. For example, in light of our knowledge on the size, stability and charge requirements for liposomal formulations to avoid removal by the MPS, the pharmacokinetic profiles of the asODN cationic lipoplexes described by Bennett *et al.* and Litzinger *et al.* could have been predicted (120, 121).

With this knowledge and background in mind, we can list some of the characteristics which would be desirable in a liposomal formulation for asODN. The formulation should be efficient at loading asODN in order to prevent wastage of material and to reduce the doses of lipid required. The particles should have a small diameter (< 200 nm) and maintain stability in the presence of biological fluids such as serum. These characteristics will reduce uptake by the MPS and allow for long circulation times required for extravasation into diseased tissues. In addition, the carrier should be targeted by attaching ligands to internalizing receptors to allow for intracellular delivery of asODN to specific cell types. One of the primary goals of this thesis was to develop a liposomal formulation for asODN which would meet all of these requirements.

In Chapter 2, two different liposomal formulations for asODN were described and characterized. Before the wide-spread use of cationic liposomes for the delivery of asODN, neutral or anionic liposomes were used to passively entrap asODN. Several studies demonstrated the ability of this type of formulation to increase the activity of asODN *in vitro* (112, 113, 132). In fact, the first example of targeting asODN to a specific cell type using immunoliposomes used asODN passively entrapped within the aqueous space of neutral liposomes (113). From a theoretical standpoint, the amount of asODN which can be passively entrapped within non-cationic liposomes is limited by the aqueous interior volume of the liposomes, and will decrease with decreasing diameter.

Work published in the early 90's on this type of formulation varied widely on the loading efficiencies obtained, and the formulations were often poorly characterized with respect to diameter. Therefore, my initial efforts in developing a liposomal formulation for asODN were aimed at properly evaluating and characterizing a passively entrapped formulation with respect to diameter, loading efficiency and asODN to lipid ratio. In addition, I wanted to determine the effect of PEG-DSPE on these characteristics, since PEG-DSPE will likely be a useful component for *in vivo* applications. My results indicate that less than 10% of added 18-mer [S]-asODN can be loaded within neutral liposomes with diameters in the 200 nm range. However, the addition of PEG-DSPE at 2-5 mol% phospholipid dramatically increased the loading efficiency up to approximately 20% of added asODN. While this is an interesting result, I concluded that 20% loading was still too low to be useful since 80% of the drug will be wasted and higher doses of lipid will be required to administer a given dose of asODN compared to a more efficient formulation.

Cationic lipids associate with asODN through electrostatic interactions and therefore can be useful in formulating liposomal asODN. However, the physical characteristics of cationic lipoplexes result in a poor pharmacokinetic profile for these formulations (120, 121). The extensive distribution to the liver is probably a result of the large diameters and positive charge attracting serum proteins. Some cationic lipid will probably be required, since utilizing the electrostatic interaction between asODN and cationic lipids is currently the only way to efficiently load asODN within liposomal carriers. Mixing pre-formed cationic liposomes with asODN may not be the best method of assembly, since it will not result in efficient charge coupling without significant aggregation. Optimizing the charge coupling between the asODN and cationic lipid could allow for efficient loading, without excess positive charge which promotes adsorption of serum proteins and increases accumulation in the MPS.

Reimer *et al.* described an extraction procedure for characterizing cationic lipid binding to plasmid DNA (pDNA) (110, 111). It involves performing a Bligh and Dyer extraction to extract pDNA from an aqueous phase into an organic phase using cationic lipids. While the purpose of the study was to characterize the stoichiometry and kinetics of the extraction, they also alluded to the potential for using the hydrophobic particles as intermediates in the formation of lipid-based DNA carriers. I also found this procedure to be useful in optimizing the charge interactions between asODN and cationic lipid. Furthermore, I envisioned that the hydrophobic particles produced may be quite similar to the inverse micelles which are produced as intermediates in the formation of REV as described by Szoka and Papahadjopoulos (119). By adding neutral 'coating' lipids to the organic phase following extraction, and then adding a small volume of aqueous phase, followed by sonication and solvent evaporation, coated cationic liposomes (CCL) are formed. As the organic phase is evaporated, the inverse micelles of neutral lipid will coat the cationic lipid-asODN particles through the hydrophobic interactions of the acyl chains. As the solvent is further evaporated, the inverse micelles of neutral lipid will destabilize, releasing the entrapped water so that the system inverts into an aqueous phase (Figure 2.5D). This is similar to the proposed mechanism of formation of REV (119).

Chapter 2 describes the procedure which was developed to produce CCL. My results indicate that CCL can be extruded to diameters under 200 nm, entrap 80 - 100% of the added asODN, and are stable in serum. The results in Chapter 3 indicate that CCL display long-circulating pharmacokinetics with less uptake into the MPS than is observed with typical cationic lipid-asODN formulations. Surprisingly, the only major difference between the pharmacokinetics and biodistribution of the CCL formulation compared with a neutral lipid formulation was a 30% uptake into the liver immediately upon injection compared to only 10% for neutral liposomes. The MPS has a high affinity for particulate drug carriers which will result in a certain amount of accumulation of almost any formulation in the liver. This is why 10% of the injected dose of asODN within neutral liposomes immediately accumulated in the liver. The increased liver uptake observed for the CCL formulation may be a result of a subpopulation of liposomes which carried slightly more positive or negative charge leading to increased opsonization and recognition by the MPS.

One of the objectives in producing CCL was to neutralize efficiently the positive charge from the cationic lipid with the negative charge from the asODN in order to reduce adsorption of serum proteins and binding to non-target cells. However, without excess positive charge, binding to target cells will also be significantly reduced. This is where targeting can play a role. The results of Chapter 4 indicate that targeted CCL can be produced by attaching targeting ligands to the surface. This was shown to increase the amount of cell-associated asODN by more than 3-fold, compared to CCL without a targeting ligand. This result indicates that the interaction of cationic lipid-containing liposomes with cells can be controlled through the use of a targeting ligand.

The main purpose of Chapter 4 was to evaluate the activity of CCL and targeted CCL in delivering active asODN. In a 'perfect world' a robust and sensitive antisense model would have provided a useful tool with which to measure the effectiveness of CCL formulations compared to standards such as LipofectAMINE® or free asODN. The next step would be to move into therapeutic experiments in an *in vivo* model, and this was always a consideration in choosing cell lines and potential genes to target. Unfortunately, the field of antisense therapeutics does not exist in a 'perfect world', and this has been well-documented in the literature, as well as in this thesis (26, 209-211).

The first gene which I chose to target was the hepatitis B virus (HBV) surface antigen (sAg) gene which was stably transfected, along with the rest of the viral genome, into a human hepatocellular carcinoma cell line (HEPG2/2.2.15) (213). While none of the results from this work have been included in the thesis, they are worth discussing briefly. The HEPG/2.2.15 cells express the asialoglycoprotein receptor which can be targeted by attaching asialofetuin onto the surface of liposomes or other drug carriers (214-217). There are examples in the literature of inhibition of HBV sAg gene and other HBV genes using asODN (41, 218, 219). Attempts to inhibit the HBV sAg gene using free asODN or asODN complexed with cationic lipoplexes, both non-targeted and targeted with asialofetuin, were unsuccessful. The reason for a lack of success is unclear, but interestingly, at least one other group has cited problems targeting HBV in HEPG2.2.15 cells. Gao *et al.* used the HEPG2.2.15 cell line in a study of the *in vitro* uptake and subcellular distribution of asODN and in the manuscript the authors indicated that they had attempted to inhibit the expression of several different HBV mRNAs using asODN, but were unsuccessful (177).

The next gene I chose to target was *MDR1* gene which codes for P-gp and is responsible for the multidrug-resistance (MDR) phenotype in many different forms of cancer. As reviewed in Chapter 4, *MDR1* has been shown to be a good target for antisense inhibition in several different studies. An MDR cell line was created by transfecting a human B-lymphoma cell line (Namalwa) with the *MDR1* gene and then selecting for transformants in culture medium containing vincristine (work performed by Susan Cubitt in the Allen Lab). The Namalwa/MDR$_{40}$ cells are maintained in medium containing 40 ng/ml vincristine, a concentration which immediately inhibits the proliferation of non-transfected cells. The Namalwa/MDR$_{40}$ cells appeared to represent a good model in which to evaluate antisense inhibition of *MDR1* for several reasons. The level of resistance is more clinically relevant than many other established MDR cell lines; there is only a 10-20 fold difference in the 1 hour IC$_{50}$ for doxorubicin in these cells compared to the parent cells. In addition, Namalwa/MDR$_{40}$ cells express the CD19 receptor, which has been shown to internalize upon binding by αCD19 mAb, providing an active route of intracellular delivery for asODN formulated in αCD19-CCL (Figure 5.1).

However, a model in which the level of mRNA was more directly correlated to the level of protein, and a definite link existed between protein levels and phenotype, may have been more sensitive. There appears to be some inconsistency between the level of *MDR1* mRNA and the level of P-gp, possibly as a result of P-gp having a long metabolic half-life. In addition, there may have been other mechanisms of resistance which would prevent us from changing the phenotype significantly by only targeting *MDR1*.

An inducible system in which gene expression can be controlled by the addition of a drug (e.g. the tetracycline-controlled transactivator (220)) or a cytokine may be more appropriate. In this case there would be a definite link between mRNA, protein and phenotype, making results easier to interpret. In addition, background expression could

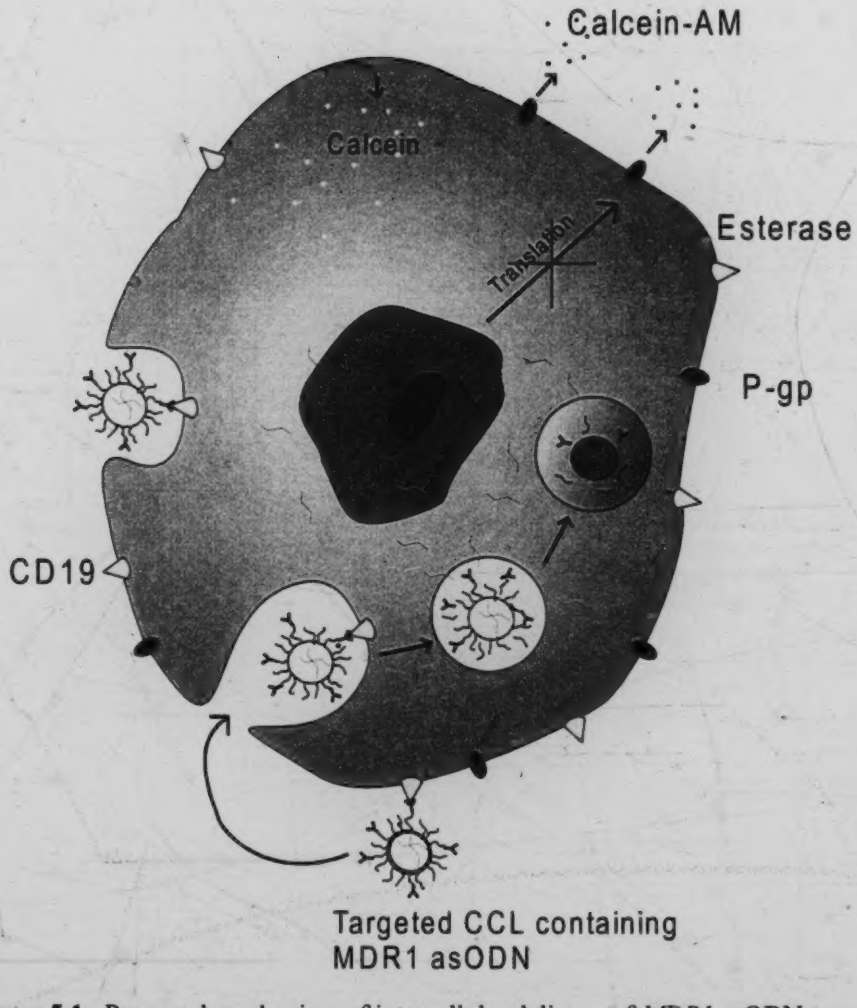

Calcein-AM
Calcein
Translation
Esterase
P-gp
CD19
Targeted CCL containing MDR1 asODN

Figure 5.1. Proposed mechanism of intracellular delivery of *MDR1* asODN to Namalwa/MDR$_{40}$ cells using αCD19-targeted CCL. Binding to CD19 will result in receptor-mediated endocytosis. Once inside endosomes or early lysosomes asODN will have to be released order to hybridize *MDR1* mRNA in the cytoplasm or nucleus. Inhibition of *MDR1* translation should result in decreased levels of P-gp on the cell surface. Therefore, asODN-treated cells should retain more calcein or doxorubicin resulting in increased fluorescence or cytotoxicity.

be low providing a very sensitive model in which to evaluate different liposomal asODN formulations. Interestingly, the ICAM-1 model which is being evaluated in clinical trials and has been mentioned throughout this thesis is an inducible system (94). Endothelial cells, keratinocytes, fibroblasts, and leukocytes normally express low levels of ICAM-1. Expression of ICAM-1 is inducible by several cytokines including interleukin-1β, tumour necrosis factor-α, and interferon-γ (94). *In vitro*, endothelial cells can be treated with ICAM-1 asODN using Lipofectin for 7-8 hours, and then ICAM-1 is induced by adding one of the cytokines listed above (94). ICAM-1 protein is maintained at background levels by asODN treatment because the newly transcribed mRNA is inactivated by the already present asODN. Control cells have approximately 100-fold higher levels of ICAM-1 16 hours following cytokine stimulation . This appears to be a very sensitive model which may be useful for evaluating different delivery strategies for asODN.

Initial experiments in which I evaluated *MDR1* mRNA levels in Namalwa/MDR$_{40}$ cells following treatment with asODN *in vitro* were somewhat promising. Treatment with asODN formulated in αCD19-CCL, non-targeted CCL, LipofectAMINE®, or a high concentration of free asODN (20 μM) resulted in subtle decreases (10 - 30%) in *MDR1* mRNA, relative to the sODN treated controls. At the same time, a functional assay for P-gp activity, the calcein-AM retention assay suggested that the asODN formulated in αCD19-CCL was causing a significant decrease in P-gp activity. The other asODN-treated groups did not demonstrate statistically significant decreases in P-gp activity.

From a therapeutic standpoint, the most important test for *MDR1* antisense activity is the ability to decrease the resistance to a chemotherapeutic agent such as doxorubicin. Therefore, the ability of asODN to decrease the IC_{50} for doxorubicin in the Namalwa/MDR_{40} cells was evaluated. Unfortunately, asODN treatment was not able to change the degree of resistance. These results contradict the results for the calcein accumulation assay; however, there are at least two reasonable explanations for this. The calcein-AM accumulation assay could be considered a more direct assay for P-gp activity, since it measures pump activity over a very short time (15 minutes) and therefore is not affected by potential changes in gene expression or cell proliferation. In short, this assay could have detected changes in P-gp levels which would not be detected in a cytotoxicity assay. The alternate explanation is that the observed increases in cellular fluorescence are relatively small, compared to the difference in cellular fluorescence of the Namalwa/MDR_{40} cells versus the Namalwa/Parent cells. This may be an example where statistical significance does not translate into biological significance.

I used an *MDR1* antisense sequence which was previously shown to be active (150). The asODN targeted the region of the initiation codon which has been shown to be sensitive to antisense treatment in several other studies (reviewed in (190)). In retrospect, this may not have been the best approach, and several sequences should have been evaluated. The studies cited above which inhibited *MDR1* expression by targeting the initiation codon used different cell lines which were either selected for

MDR1 overexpression from the genomic DNA, or were transfected with other *MDR1* cDNAs. Northern analyses indicate that the Namalwa/MDR$_{40}$ cells which I used produce a unique *MDR1* mRNA. While the sequence around the initiation codon as well as the entire *MDR1* sequence may be the same, the mRNA contains coding sequences from the vector (pHAMDR/A). Therefore the secondary structure of the mRNA is different and could inhibit asODN from hybridizing to the initiation codon. If a series of sequences was screened, a more active asODN may have been found.

The difficulties encountered in attempting to use asODN to decrease the expression of specific genes, and ultimately modulate the phenotype of target cells are hardly surprising in light of the controversy which has surrounded the antisense field. The results of this thesis leave the question about the utility of CCL to deliver asODN largely unanswered. Fortunately Dr. Allen has established a collaboration with Dr. Mirco Ponzoni (G. Gaslini Children's Hospital, Genoa, Italy) who has considerable experience with the use of asODN. I provided them with a detailed protocol for making CCL and antibody-targeted CCL as well as measuring the cellular uptake of asODN by the slot blot method. They were able to produce antibody-targeted CCL and test a c-myb asODN against a human neuroblastoma (NB) cell line *in vitro*. The proliferation of NB cells is dependent on c-myb expression. The NB cells also express disialoganglioside GD$_2$, which is expressed by several different tumours. Binding of GD$_2$ by an anti-GD$_2$ mAb results in receptor-mediated endocytosis and therefore these cells can be targeted by attaching an anti-GD_2 monoclonal antibody onto the surface of CCL. Treatment with anti-GD_2 CCL resulted in significant increases in uptake of asODN by NB cells. Results indicate that asODN formulated in anti-GD_2 CCL was more effective than asODN formulated in non-targeted CCL or free asODN at inhibiting the proliferation of NB cells. In addition, GD_2-CCL containing c-myb asODN specifically inhibited the proliferation of NB cells in a mixed cell population (221).

Although the data presented in Chapter 4 did not provide a clear answer on the effectiveness of the CCL formulation, or the targeted CCL formulation, their activity was confirmed in the NB/c-myb model.

In spite of the lack of definite antisense activity obtained in my experiments, further work using this carrier system is worth pursuing, especially in light of the results recently obtained by our collaborators. Antisense technology will continue to develop and in the near future we will probably see more examples of asODN being used clinically. Liposomes, may prove useful in some of these applications. As alluded to earlier, the ICAM-1 asODN which is in clinical trials for inflammatory conditions (Crohn's disease, rheumatoid arthritis) may be a good candidate for testing the efficacy of a liposomal carrier. Liposomes have recently been shown to accumulate at sites of inflammation such as arthritic joints (222) as well as inflamed bowel in an animal model of acute colitis (223). It seems plausible that liposomes could increase delivery of the ICAM-1 asODN to inflamed bowel, or joints. Another attractive feature of this model is that the University of

Alberta Hospitals is one of the sites participating in the Crohn's disease clinical trial, providing the potential for a readily available collaboration. Finally, another possible application for a liposomal formulation of the ICAM-1 asODN would be in psoriasis. The sterically stabilized liposomal doxorubicin formulation, Caelyx®, shows preferential accumulation in skin lesions of AIDS patients with Kaposi's sarcoma. Therefore, similar liposome formulations may be able to deliver more ICAM-1 asODN to patches of skin affected by psoriasis.

In summary, the results of this thesis support the hypotheses set out in the Introduction. Cationic lipids were used in a liposomal formulation of asODN which has a small diameter (< 200 nm), it is efficient at loading asODN, and it is stable in plasma. Intravenous administration of CCL results in long circulating pharmacokinetics with significantly less uptake into the liver compared to typical cationic lipoplexes. Results also indicate that *in vitro* delivery of asODN using CCL is significantly reduced compared to cationic lipoplexes, however, delivery could be significantly increased by attaching a targeting ligand onto the surface. Although the activity of the CCL formulation *in vitro* was confirmed by our collaborators, I was not able to evaluate the activity of the CCL formulation *in vivo* because I did not have a suitable therapeutic model. Therefore, the question of the utility of liposomal carriers for asODN *in vivo* remains largely unanswered.

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